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Disposable Bioreactors

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Preface

Over the past five years, the immense financial pressure on the development and manufacturing of biopharmaceuticals has resulted in the increasing use and acceptance of disposables, which are discarded after harvest and therefore intended only for single use. In fact, such disposables are implemented in all the main bioprocess production stages today and an even higher growth than those in the biopharmaceutical market is predicted (reaching double figures). Alongside disposable filter capsules, membrane chromatography units, tubing, connectors, flexible containers processing or containing fluids, freezer systems, mixers and pumps, and fully controlled disposable bioreactors of up to 2,000 L culture volume are already available on the market.

Numerous studies highlight the advantages of disposable bioreactors and reveal their potential for simple, safe and fast seed inoculum production, process development and small as well as middle volume production (e.g. bioactive substances, viruses for vaccines and gene therapies etc.). They suggest that such disposable bioreactors (typically characterized by the cultivation chamber or bag from plastic materials) may be advantageous for plant, animal and microbial cells. Running industrial activities such as CFD-modelling, development of single-use process monitoring and control technology, and standardized film formulations are attempting to resolve the limitations of the current disposable bioreactors. These achievements, along with substantial improvements in product yield, will reduce the use of stainless steel in the biomanufacturing facilities of the future.

The aim of this volume, which includes eight contributions from renowned experts, is to give an up-to-date overview of the main disposable bioreactors, their working principles, characteristics, known engineering aspects, and potential applications. R. Brecht discusses disposable bioreactor technology development in pharmaceutical glycoprotein manufacturing from the view of the applier (CMO). The advantages of orbital shaken disposable bioreactors from millilitre-to 1 m^3-scale are outlined by X. Zhang, M. Stettler, D. De Sanctis, M. Perrone, N. Parolini, M. Discacciati, M. De Jesus, D. Hacker, A. Quarteroni and F. Wurm for animal cell culture-based processes. R. Eibl, S. Werner and D. Eibl focus on a wave-mixed bag bioreactor which, to a large extent, promoted the development of disposable bioreactors. In this context they summarize engineering aspects which provide invaluable information to influence the cultivation procedure positively and

thereby, the results of cultivations with bioreactors based on wave-induced motion up to 120 L culture volume. The importance of disposable bioreactors for liver tissue engineering and the resulting transport phenomena are described by G. Catapano, J.F. Patzer II and J.C. Gerlach, whereas three new types of disposable bioreactors, which have been developed to cultivate plant cell suspension cultures and somatic embryo cultures, by researchers at Nestlé (J.P. Ducos, B. Terrier, D. Courtois) are presented.

Furthermore, the consideration of several aspects closely connected with bioreactors will facilitate readers in deciding for or against disposable alternatives and help them to choose an appropriate system. For the same reason, the availability of disposable sensors for process monitoring and control, as well as suitable equipment for disposable downstream processing, influenced by a bioreactor's cultivation result, are discussed by A. Glindkamp, D. Riechers, C. Rehbock, B. Hitzmann, T. Scheper and K.F. Reardon, and U. Gottschalk. Finally, the configuration of hybrid and disposable production facilities is addressed by A. Ravisé, E. Cameau, G. De Abreu and A. Pralong.

This volume constitutes a reliable resource book, which gives due attention to the most pressing problems to guarantee desired product yields, in combination with product consistency and economic benefits in processes mainly aimed at the production of cells and bioactive substances, using disposable bioreactors and disposable equipment (also partly for downstreaming). The editors are grateful for the support of all the contributors, the series editor Prof. T. Scheper and the publisher, who have made this book possible.

Summer 2009   Regine Eibl
                                                                                          Dieter Eibl

Contents

Disposable Bioreactors: Maturation into Pharmaceutical Glycoprotein Manufacturing

René Brecht

Abstract Modern biopharmaceutical development is characterised by deep understanding of the structure activity relationship of biological drugs. Therefore, the production process has to be tailored more to the product requirements than to the existing equipment in a certain facility. In addition, the major challenges for the industry are to lower the high production costs of biologics and to shorten the overall development time. The flexibility for providing different modes of operation using disposable bioreactors in the same facility can fulfil these demands and support tailor-made processes.

Over the last 10 years, a huge and still increasing number of disposable bioreactors have entered the market. Bioreactor volumes of up to 2,000 L can be handled by using disposable bag systems. Each individual technology has been made available for different purposes up to the GMP compliant production of therapeutic drugs, even for market supply. This chapter summarises disposable technology development over the last decade by comparing the different technologies and showing trends and concepts for the future.

Contents

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Abbreviations

ADCC	Antibody derived cytotoxicity
ASTM	American society for testing and materials
BPC	Bioprocess container
CAGR	Capacity annual growth rate
CAPEX	Capital expenditures
CHO	Chinese hamster ovary
CIP:	Cleaning-in-place
CMO	Contract manufacturing organisation
COGs	Cost of goods
DO	Dissolved oxygen
EP	European pharmacopeia
EPO	Erythropoietin
EVA	Ethylene-vinyl-acetate
EVOH	Ethylene-vinyl-alcohol
FDA	Food and drug administration
GMP	Good manufacturing practice
ICH	International conference on harmonisation
IMP	Investigational medicinal product
ISO	International organisation of standardisation
ISTA	International safe transit association
JP	Japanese pharmacopeia
mAb	Monoclonal antibody
MCB	Master cell bank
MEM	Minimum essential medium
PAT	Process analytical technology
PE	Polyethylene
S.U.B.	Single use bioreactor
SBA	Summary basis of approval
SBBSlug	bubble bioreactor
SIP	Steam-in-place
USP	United states pharmacopeia
WCB	Working cell bank
WFI	Water for injection

1 Introduction

Complex biological drugs are increasingly found in the pharmaceutical industry product pipeline. Over 350 approved biologics and more than 500 proteins in different stages of development [1] prompted the biotechnology industry to find solutions for flexible, cost efficient and scalable bio-manufacturing.

To fulfil these demands, the used host cell was diversified and the expression level was dramatically increased by the optimisation of vector elements and effective clone selection procedures as well as by the improvements in medium and feed composition and in the overall fermentation strategy. As a result, the volumetric productivity has been increased 100-fold over the last decade [2], and the bottleneck is moving downstream.

On the other hand, a deep understanding of the structure activity relationship of therapeutic proteins has been developed, especially of glycoproteins that are produced by mammalian cells. The capabilities of controlling the activity as well as the pharmacokinetic profile of protein drugs have become a key issue in drug development. As an example, the removal of the core fucosylation from the glycosylation moiety of monoclonal antibodies led to a remarkable increase of the antibody derived cytotoxicity (ADCC) [3], and is expected to be a powerful and elegant approach to the design of the next generation of therapeutic antibodies with improved efficacy [4].

The combination of process improvements with a higher drug efficacy will finally result in a decline of the required production capacity for the same amount of active product. Nevertheless, the market demand, especially for monoclonal antibodies, is still growing. Thus, the industry-wide expansion of capacities over the last decade was mainly driven by antibody requirements.

In general, the terms of capacity can be distinguished between high volume, low potency products (e.g. monoclonal antibodies with doses ≥100 mg) and low volume, high potency products (e.g. cytokines with doses <1 mg).

Figure 1 gives an overview of the recombinant protein drugs, comparing their annual demand and sales in 2005. It becomes obvious that high potency drugs are very attractive and highly susceptible to the development of biosimilars and second generation products. In addition, several first generation drugs have already been approved for some indications competing with each another for market share.

The major drivers in the competition process are, besides the comparability or even superiority in the pharmaceutical efficacy, the production costs and the overall development time. Flexibility in capacities and capabilities to develop customised processes in an accelerated time schedule are highly needed.

Options for providing different modes of operation using disposable bioreactors in the same facility can fulfil most of these demands and support tailor-made processes. Due to the efforts in disposable technology development and the ongoing improvements in the production yield, these processes will be scalable also for market supply for a variety of recombinant proteins in the near future.

The use of disposable bioreactors and, finally, the development of integrated disposable based production processes are key issues in current biopharmaceutical manufacturing.
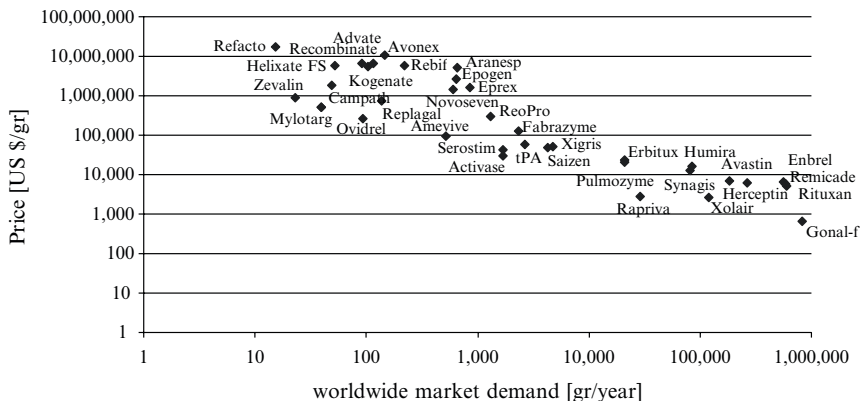
Fig. 1 Prices vs. market demand of approved glycoprotein and monoclonal antibody drugs as reported for 2005

2 Disposable Bioreactor Technologies

2.1 History

The first synthetic fibre was developed in 1884 from cellulose acetate. The history of plastic made from synthetic components started with the synthesis of Bakelite by Belgian American Dr. Leo Baekeland in 1909. It was formed by the pressure and heat driven reaction of phenol with formaldehyde. It was followed by the production of polyvinyl chloride (1912), polyethylene (1933) and polypropylene (1957) among many other polymers. By the 1960s, glass syringes which were re-useable after sterilisation were being replaced with disposable plastic syringes and single-use needles. The first type of plastic chosen for this use was polystyrene. The idea of avoiding the transmission of infections by immediate disposal after use became reality. High throughput at low cost by using disposable syringes has proven to be of advantage in the vaccination of large populations.

By the 1980s, disposable devices such as filters, tubing, bags, bottles and syringes entered the biotechnological manufacturing environment.

In 1989, the recombinant glycoprotein Erythropoietin (EPO) from Amgen Inc. was approved by the FDA for the treatment of anaemia. The production process is based on the cultivation of CHO cells in a large number of roller bottles.

Since 1972, hollow fibre cartridges, used primarily as artificial kidneys for dialysis patients, were applied to cultivate cells to tissue-like densities [5].

The first recombinant protein produced in hollow fibre bioreactors was approved in 1996 by the FDA. Capromab pendetide (Prostascint®) from Cytogen Corp. is indicated as a diagnostic imaging agent in newly-diagnosed patients with biopsy-proven prostate cancer.

However, the scale-up of hollow fibre bioreactor systems has technical and economical limits. So far, the largest available system can operate approximately 2.5 L of culture volume. Despite the high cell density inside, the cartridge volume is limited to 110 mL and the scale-up is realised only by running up to 20 cartridges in parallel.

The breakthrough in disposable bioreactor technology development in terms of larger capacities was the use of bag systems as culture ware.

The WAVE bioreactor System20 became commercially available in 1998 and the technology began its triumphant success. The largest system today has a bag size of 1,000 L corresponding to a working volume of 500 L. Almost half of the bag size is filled with air, and therefore no active oxygen supply is needed.

A higher utilization rate of the bag size as cultivation space was realised by the development of single-use stirred-tank bioreactors. Due to the active oxygen supply and the different mixing principle, nearly the whole bag volume can be filled up with medium. The SUB (single use bioreactor) from HyClone entered the market in 2006. Actually, disposable stirred-tank bioreactors up to 1,000 (HyClone) and 2,000 L (Xcellerex) culture volume are commercially available and the plans are to develop 3,000-L bioreactor systems over the next couple of years. These systems should be suitable even for the commercial production of a large variety of therapeutic recombinant protein products. Nevertheless, the comparable mixing principle also allows a seamless tech transfer to large-scale stainless steel facilities with up to 20,000 L vessel volume, especially for the production of high volume monoclonal antibody products.

The current available disposable bioreactor technologies are the key to success for pilot plant scale to fulfil the fast growing demand for flexible multi-product facilities that enable the development and production of several recombinant protein drugs at the same site.

2.2 Technology Overview

It is preferable to distinguish the different disposable bioreactor technologies according to their differences in the mixing principle or the culture ware used.

Bioreactor systems using bags as culture ware can be divided into mechanically (tipping, stirring, vibrating) and pneumatically driven (airlift, bubbles) devices [6]. The mechanically driven bioreactors with an integrated stirring system or with wave agitation are most advanced for the biopharmaceutical fermentation of mammalian cells. These devices are mainly used for batch and fed batch fermentation processes. Nevertheless, the equipment can be extended to retain the cells and to use them also for perfusion. The higher comparability to the mechanically driven, stainless steel vessels lowered the market's entry hurdles. In terms of capacity, the systems are not only suitable for early material production but also for the market supply of low volume products.

The pneumatically driven systems such as the newly introduced SBB (Slug Bubble Bioreactor) from Nestlé and the older Plastic Lined Bioreactor, are reported

to be used for plant cell cultivation [7]. Both use a bubble column up to a total culture volume of 100 L.

More flexibility in the mixing principle, leading to a broader application spectrum, is offered by the CellMaker system from Cellexus. This system can be operated as an airlift bioreactor alone or in combination with an additional interior stirring system for mammalian cell culture.

A totally different approach is realised by the hollow fibre bioreactors. The cells grow to high densities inside a cartridge and can be kept viable over a long period of time via medium perfusion through the hollow fibres. The integration of a pressure cycling system in the newer AcuSyst systems from BioVest dramatically increased the mass transport through the membrane and, therefore, the cell behaviour inside the cartridge. Despite the technology which has been in use for cell culture since the first experiments in the early 1970s, the technology has never developed with the same power as stainless steel technology or, later on, the mechanically driven bag systems. Their use is, therefore, still limited to niche applications.

As well as the larger production systems, there are many small cultivation devices on the market that are intended to be used during the pre-culture process, for screening experiments or even for production purposes by using them in parallel.

Small bioreactor systems, especially with two dialysis membranes to separate the culture space from the medium reservoir, are characterised by high volumetric productivity. The miniPERM system (Vivascience) and the CellLine (Integra) are very efficient for lab scale production of monoclonal antibodies up to several hundred milligrams.

Roller bottles and multi-tray bioreactors are suitable even for commercial production (e.g. Erythropoietin) by using thousands of them in parallel. Industrial automation, like the RollerCell technology from Cellon, replaces the efforts in manual operations.

Disposable spinner flasks (Corning) are small-scale stirred systems that can be used for early process development, especially for medium and feed screening. Even smaller systems, e.g. shaker tubes from Excellgene, are very appropriate for screening huge amounts of cell clones regarding volumetric productivity in different media. The combination of cell line development and process development is not only recommended to save development time, but also to find the right clone that is suitable for production. Excellgene is actually in the process of scaling up the principle of orbital shaking up to 1,000 L. The market launch of the system is expected soon.

To summarise: the range of disposable bioreactors available on the market offers flexible, cost efficient and time-saving solutions from early process development to large-scale production. Table 1 gives an overview of the different disposable bioreactor systems that are commercially available and used for mammalian cell culture.

In the following sections, three different disposable technologies that are advanced in terms of capacity, application to glycoprotein production and GMP compliance are described in more detail. The focus is on the working principle, the potential process mode and the concept of scalability.

2.3 Wave Bioreactors

The first Wave bioreactor was developed in 1996, and the technical application of commercially available devices started almost 10 years ago. The core unit consists of a pre-sterilised bag as a cell culture chamber and a rocking platform as a bag

Table 1 Overview of the different disposable bioreactor systems for mammalian cell culture

Categorisation		Bioreactor system	Company	Largest scale
Bag bioreactors	Wave agitated systems	BioWave	Wave Biotech AG Switzerland	BioWave 600, 300 L culture volume
		BIOSTAT CultiBag RM	Sartorius Stedim Biotech S.A.	Biostat CultiBag RM 50, 25 L culture volume
		Wave Bioreactor	Wave Biotech (USA), now part of GE Healthcare	System1000, 500 L culture volume
		Tsunami Bioreactor	CatchMabs	Maxi Tsunami Bioreactor 6 × 160 L culture volume
		AppliFlex Single Use Bioreactor	Applikon	AppliFlex system 50-L Bag
		CELL-tainer	Cellution Biotech	CELL-tainer CT-20, ~15 L culture volume
		Optima	Metabios	Optima DC-20, 8 L culture volume
		OrbiCell	Metabios	10 L culture volume
	Disposable stirred-tank bioreactors	Single Use Bioreactor (S.U.B.)	HyClone (now Thermo-Fisher)	1,000 L
		XDR Disposable Bioreactor	Xcellerex	2,000 L
		Nucleo (magnetic driven centrifugal pump)	Artelis/ATMI LifeSciences	25–50 L
	Hybrid bioreactor (airlift and stirring)	CellMaker Plus	Cellexus Biosystems	8 L
	Bioreactor with vibromixer	Bio-T bag	Zeta	1,875 L
Hollow fibre bioreactors	With pressure cycling	AcuSyst systems	BioVest	XCellerator with 20 cartridges, ~2.5 L culture volume
	Without pressure cycling	AutovaxID	BioVest	One cartridge, ~ 110 mL culture volume
		FiberCell	FiberCell Systems	FibreCellDuet, 2 × 150 mL cartridges

Table 1 (continued)

	Categorisation	Bioreactor system	Company	Largest scale
Other systems	Membrane bioreactors	CeLLine	Integra Biosciences	CeLLine 1000, 20 mL culture volume
		miniPERM	In Vitro Systems and Services	miniPERM classis, 35 mL culture volume
	Roller flasks	CELLroll	Integra Biosciences	Up to 32 roller bottles
		RollerCell	Cellon	RollerCell Max with up to 20 rotors (each with 20 roller bottles)
	Perfusion bioreactors	CellCube	Corning	Cell growth area 85,000 cm^2, 6 L recommended medium volume
	Multi-tray bioreactors	Nunc Cell Factory	Nunc	40 chamber CF (25,280 cm^2), 8 L recommended medium volume
		HYPERFlask	Corning	10 layers (1,720 cm^2)
	Spinner flasks	Disposable Spinner Flasks	Corning	500 mL
	TubeSpin bioreactors CultiFlask bioreactor	Shaker Tubes	Excellgene Sartorius-Stedim	50 mL tubes
	'Artificial lung'	BelloCell	Cesco	BelloStage 3000, 4 bottles × 500 mL culture volume

holder with an integrated heating system. Single use sensors for pH and DO measurement are integrated into the bag design of the newer systems and can be connected with an independent control unit.

The use of only one fixed axle allows the rocking motion back and forth as illustrated in Fig. 2. This movement generates waves at the liquid-air interface. The principal idea and application for small scale cell culture was reported even earlier [8].

The mechanism ensures a gentle mixing of the cells as well as the oxygen transfer from the gas to the liquid phase. The mixing is very efficient and it takes only a few seconds to get homogeneity in a 10-L bioreactor [9].

The bag can be only partly filled with culture medium (up to 50%). The other half of the bag is needed to ensure a nearly bubble-free aeration of the culture medium via surface exchange. The composition of the gas phase can be controlled by using a gas mixing system. The aeration is sufficient for a variety of mammalian and also insect or plant cells, but it cannot sufficiently fulfil the high metabolic oxygen
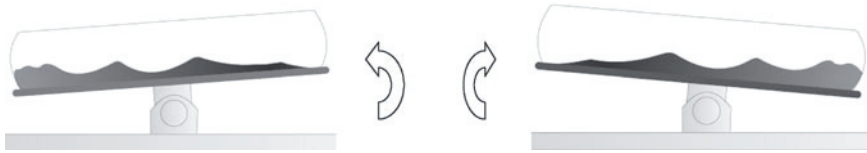
Fig. 2 Illustration of the wave motion inside the culture bag [11]

demand in microbial fermentation. Even for mammalian cell culture, the volumetric oxygen mass-transfer coefficient is a limiting factor in scale-up [10]. The high shear sensitivity of mammalian cells prevents the use of a higher velocity.

After filling the bag partly with medium, the remaining volume is inflated with the process gas mixture via a sterile inlet filter that is pre-attached to the bag. The excessive gas can exhaust via another sterile filter. An integrated backpressure control valve ensures that the bag is fully inflated at any airflow.

To avoid excessive foam formation, the bag design as well as the recommended rocking angle and rocking speed were carefully tested and optimised. The rocking angle and the number of rocks per minute have to be determined for each volume used in the bag. Interestingly, smaller bag volumes require a higher rocking angle than larger ones to ensure the same mass transfer characteristics [11]. In principle, the rocking angle in the Wave bioreactor 200 can be adjusted between 4° and 12° and the rocking rate between 5 and 25 rocks per minute.

The bag design includes ports for sterile sampling and harvesting as well as for the addition of feed or buffer solutions. The newer BIOSTAT CultiBag system with optical package allows online monitoring and control of pH and DO using optical single-use probes. However, the pH value especially has to be recalibrated at certain intervals due to a slightly probe-related shifting process over time.

The temperature is controlled by a non-invasive temperature sensor integrated into the rocking platform. The heater inside the base plate warms the underside of the bag. Due to the efficient mixing strategy, there is no temperature gradient inside the culture volume.

The measuring and control unit enables the regulation of pH, DO, temperature, rocking rate and gas flow and allows complete process documentation, especially required for GMP production purposes.

In terms of capacity, the largest system offered by GE Healthcare has 1,000 L bag size. According to the company's presentation at the BioProduction conference in October, 2007, in Berlin, this system can operate up to 650 L working volume. The System 600, offered by the Swiss Wave Biotech AG works with up to 300 L culture volume.

In principle, there are two options for the scale up process. The first one increases the bag size. An alternative is offered by using the Tsunami Bioreactor system that runs several individual bags in parallel.

For the induction of an identical wave motion, the depth and height of each culture bag, and in particular the ratio between depth and height, have to be kept constant for all bags. The liquid height level can be maintained identically only by

varying the width of the bags. To get comparable results the filling percentage should also be the same.

Nevertheless, the scale up to the larger scale Wave bioreactor systems with 200–1,000 L bag volume requires an increase of culture bag size in all three dimensions. The changed wave hydrodynamics influence gas exchange and growth conditions.

The oxygen transfer rate especially is a critical parameter in the scale up process. To keep it on an equal level, the rocking angle and the rocking rate have to be adapted for each individual bag size. Thus, a higher rocking angle and rocking rate are needed for the smaller bag sizes to get the same mass transfer characteristics as the bigger ones.

A linear scalability is offered by a parallel design of the assembly. When increasing the number of platforms or increasing the width of the assembly, neither the rocking angle nor the rocking rate has to be changed. This concept is realised in the Tsunami Bioreactor. Only one electric motor and speed-regulatory unit is required. The entire assembly has an efficient small footprint, especially when more than one platform is placed on top of another along a vertical axis. Each platform rocks in counter phase to its adjacent platform to reduce engine power and provide maximum stability. The overall design is based on a multilayer wave agitation. Fixed height and depth for the bag sizes ranging from 5 to 160 L ensure identical hydrodynamics [12]. The optimised conditions, therefore, from a 64 × 5 L bag matrix can be easily transferred to the 160-L scale. Besides the disposable concept, this further reduces development time and costs in the scale up process.

To increase the batch productivity without increasing the bag size, the Wave bioreactor can also be adapted to run in a perfusion mode. For this purpose, a unique floating filter that is installed inside the bag system has been developed by Wave Biotech. The filter floats on the surface of the liquid and the wave motion prevents filter clogging. The cells are retained inside the bag. Therefore, no external pump is required for the recirculation of the cells. The perfusion rate is regulated by a weight-based perfusion controller.

The application of the system to the production of a monoclonal antibody by using hybridism cells resulted in a maximum viable cell density of $2E + 07$ cells per mL [13]. In addition, the mAb volumetric productivity (33.1 mg L^{-1} day) in perfusion culture was much higher in comparison to the corresponding batch culture (20.3 mg L^{-1} day). Another application showed that the perfusion principle could be scaled up from 25 to 500 L working volume without a negative effect on cell growth or protein production [14].

The perfusion system also opens the potential application spectrum to the cultivation of patient-specific cells at high cell densities.

In the bio-manufacturing area, the technology is currently used for glycoprotein as well as virus and vaccine production [16–18].

The Wave bioreactor was applied to mammalian cell culture using a variety of different host cells (e.g. CHO, NS0, hybridoma, HEK293, PER.C6 and also primary human cells).

The potential application in production can be divided into two segments due to capacity reasons. For high volume products, the technology is suitable as seed

train for the preparation of inoculums for process-scale cultivation in conventional stirred-tank reactors. For low volume products, such as some glycoproteins and vaccines, the whole production process can be covered by disposable wave agitated systems.

2.4 Disposable Stirred Bag Bioreactors

Another disposable bag system based technology entered the biopharmaceutical market in 2006. Due to the design of the disposable stirred bag bioreactors, the working principle is very similar to the traditional stainless steel vessels. This favours the technology in terms of comparability and process transfer to large scale facilities. Nevertheless there are differences, especially in the culture ware, the mixing principle and the temperature control.

Actually, disposable stirred-tank bioreactors up to 1,000 L (HyClone) and 2,000 L (Xcellerex) culture volume are commercially available. Both technologies provide a specially designed disposable bag culture ware that is integrated into a permanent support vessel (stainless steel). The bag design includes all gas filters, gas and liquid transfer tubes, sparger, ports for probes and sampling as well as the stirring system.

The S.U.B. (single use bioreactor, Fig. 3) from HyClone has an integrated pitch blade impeller with an angled shaft connected to the top. The shaft is stabilised by the introduction of a metal stick that has no direct contact with the cell culture space.

The vessel is equipped with an electrical heater jacket in the lower part to regulate the cultivation temperature. It takes more time to adapt the temperature to a certain level due to the reduced directly heated surface area in comparison to the conventional stirred-tank bioreactors.

Fig. 3 250 L S.U.B

The XDR Disposable Bioreactor from Xcellerex works with a magnetically coupled in-bag agitator that requires no shaft penetrations through the bag. The kettle is jacketed with multiple zones supporting 5–1 turndown. A closed loop controller ensures temperature regulation by an optional added water supply and drainage for both heating and cooling. According to the manufacturer's data, it takes 30–45 min to heat up.

Both systems, XDR and S.U.B., work with traditional controlling systems for pH and DO. There is a sterile insertion system for the probes in place.

Whereas the S.U.B. integrates with other existing bioreactor control systems, the XDR system is offered with its own Delta V or PLC control system.

The critical parameters for the scale up are the power input per unit space, the tip speed and the oxygen transfer coefficient. To ensure comparable results, the height-to-diameter working volume ratio is maintained at 1.5:1. The S.U.B. bioreactors are offered with maximum working volumes of 50, 250 and 1,000 L. The XDR Bioreactors are available with 200, 500, 1,000 and 2,000 L of working volume.

Both technologies are intended to be used preferably for mammalian cell culture. The existing systems are already sufficient in terms of capacity for the commercial production of low volume glycoprotein products. For large volume products, e.g. monoclonal antibodies, the similarities in the mixing principle and the overall bioreactor design should enable a tech transfer to large-scale stainless steel facilities with up to 20,000 L vessel volume.

The first comparability studies of the S.U.B. bioreactor technology with conventional stainless steel bioreactors were reported in 2006 [19]. The information provided by Baxter and by Centocor showed very similar results regarding viable cell density and viability profile as well as the expression profile and the analysed product quality.

Similar to the Wave bioreactor, the disposable stirred bag bioreactors have already been tested for their potential application for continuous perfusion processes. For this purpose, the bioreactor system was equipped with an external cell retention device. Xcellerex demonstrated the successful implementation of a pneumatic Centritech system at 200 L and 1,000 L scale [20]. Centocor has also applied the S.U.B. bioreactor system to a perfusion process design.

2.5 Hollow Fibre Bioreactors

2.5.1 Traditional Systems

In contrast to the systems described above, the use of hollow-fibre bioreactors is dedicated to only allow high cell density perfusion processes. Besides the roller bottles, it was the first scalable and larger production system on the market using disposable culture ware.

Nevertheless, the capacity of commercially available bioreactors is so far limited to a culture space of 2.5 L (20 hollow fibre cartridges). Therefore, their use is

restricted to the production of low volume biopharmaceuticals and in vivo diagnostics [21]. They are also suitable for producing first amounts of material without sophisticated process development. Figure 4 shows a flow path with an assembly of six cartridges.

The system is characterised by an independent medium and harvest stream. This allows retention of both cells and product in the culture chamber. The separation is realised by using hollow fibre cartridges with an intra- and extra-capillary space. The system has two independent cycling pathways. One provides a continuous flow of medium through the hollow fibres and the oxygenator (medium circulation). The other one runs through the extra-capillary space, also termed the culture space, and is used for the inoculation procedure and product harvest. Because harvesting is independent of the feeding flow, the product concentration can be adjusted [22]. This is especially advantageous for instable products, to overcome product inhibition (higher harvest rate) or to reduce the harvest volume (lower harvest rate).

For the first generation type of bioreactors, the mass transfer through the membrane is only diffusion-controlled. This can lead to insufficient oxygen supply, membrane fouling, inhomogeneous product harvest and inappropriate removal of dead cells and debris. In an attempt to avoid such problems, a specific second generation type of bioreactor was developed. Reactors of this type are equipped with an expansion chamber for each pathway in which gas pressure can be selectively increased (up to 100 mmHg). This ensures a controlled trans-membrane pressure providing a much larger mass transfer than that obtained via diffusion. An advantageous mixing effect occurs when this trans-membrane pressure, and as the result the fluid flow, is reversed. The trans-membrane flow can be adjusted as high as six times the cell culture volume per hour.
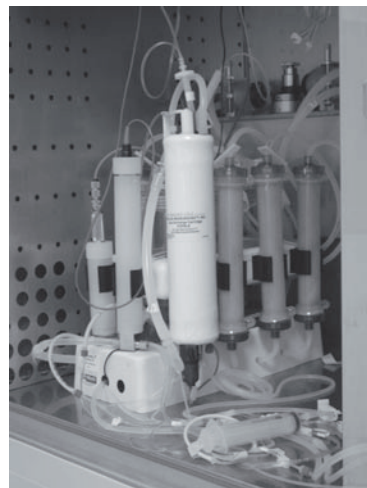
Fig. 4 An interior view of the AcuSyst X-Cell bioreactor with one flow path that operates six cartridges in parallel

The perfusion technique and the near absence of any shear stress allow the cells to grow to very high cell densities ($>10^8$ cells mL^{-1}). The cells are arranged in a tissue-like manner around the hollow fibres (as shown in Fig. 5).

It is impossible to determine the exact count of viable cells or to isolate large cell amounts from the bioreactor providing proof of batch-to-batch consistency. The performance and viability of the cells can only be monitored and prescribed by measuring metabolic activities such as oxygen or glucose consumption and expression of the protein of interest.

There is a good correlation between cell specific productivity (pg per cell and day) and the specific productivity in the bioreactor (mg per cartridge and day) that allows the calculation of the expected production amount at a very early stage of development. Nevertheless, the production is restricted to low volume products due to the limitation in scale even for high producer cell lines.

Besides their use in mammalian cell culture, hollow fibre bioreactors are also suitable for virus and viral vector production [23].

Advances in fibre materials can further improve the productivity of hollow fibre systems and open the technology to potential applications in the field of tissue engineering [24].

2.5.2 Development of a New Membrane-Based Bioreactor Generation

A new membrane-based bioreactor system was developed by ProBioGen AG in Berlin in order to overcome the limitations in scalability. The main principle of the original hollow fibres was inverted to grow the cells inside cell culture tubes. These tubes are fixed horizontally in a disposable, rotating cylindrical bioreactor vessel. During cultivation, the rotation ensures the exposure of the cells alternatively to oxygen and medium to allow a sufficient nutrient supply, waste removal and product harvest.

The rationale behind bioreactor design has been to support a continuous protein production process. This maintains a permanent cell free product harvest from a
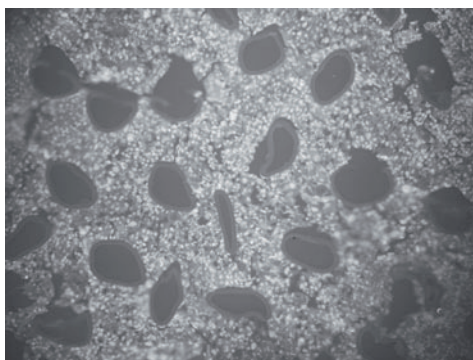
Fig. 5 Cross section through a hollow fibre cartridge filled with cells, hollow fibres in *dark grey*
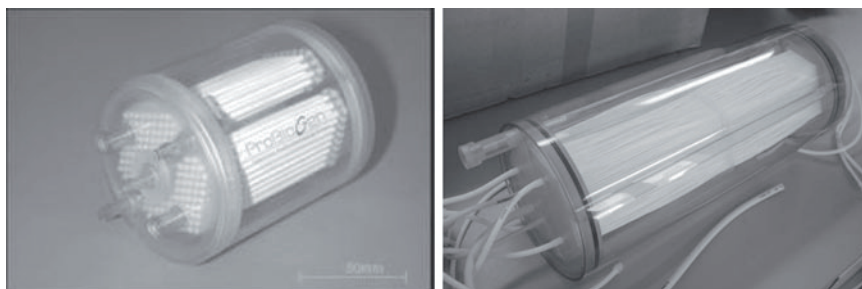
Fig. 6 Small-scale bioreactor vessel for process development (*left*) and the pilot scale bioreactor module (*right*)

high cell density culture. The cell retention goes along with highly sufficient oxygen supply and minimised shear stress [25].

By using a tube as the cell culture space a maximum distance of less than 1 mm between each cell in the system and the gas phase is realised. A membrane wall thickness of 200 mm is sufficient to support cell densities >10^8 cells mL^{-1} inside the tube.

The tube also protects the cells against shear stress which is further minimised by a gentle mixing rate.

The selected polyethersulfone microfiltration membrane has pore sizes up to 1.4 mm for reliable cell retention as well as sufficient protein passage. The surface properties prevent membrane clogging over cell culture periods of 60 days.

The new bioreactor system will operate on three different scales. The small scale device has a vessel volume of 100 mL and can run eight bioreactors in parallel for fast and efficient process development (Fig. 6).

The pilot scale bioreactor with a vessel volume of 10 L is intended to be used for preclinical and investigational clinical material production (Fig. 6).

The commercial scale bioreactor has a vessel volume of 100 L to allow market supply. A bioreactor system with 400 L vessel volume is envisaged.

The layout of the bioreactor hardware (pumps, control unit, power supply) allows the running of pilot as well as commercial scale disposable vessels. This may substantially speed up technical transfer and scale up.

3   Advantages and Challenges of Disposable Bioreactors

Current biopharmaceutical development increasingly favours the advantages offered by the use of disposable bioreactor technologies. The high level of flexibility and safety combined with low capital investment are appreciated by an industry that has to develop and produce several products at the same site at reduced costs and with a high utilisation rate of the whole bio-manufacturing plant.

The higher level of flexibility is mainly ensured by short changeover procedures and the use of portable equipment with low space requirements. The downtime especially between consecutive production runs can be reduced to a minimum of 1–2 h because it is just a simple culture ware (e.g. bags) replacing procedure [26]. In contrast to that, for traditional stainless steel bioreactors it takes 8–10 h downtime for consecutive runs of the same product and 3 weeks for a full product changeover procedure. All the cleaning and validation requirements have to be fulfilled to avoid cross contamination and to ensure sterility. Qualified cleaning-in-place (CIP) procedures are needed and the methods to demonstrate the absence of biological and cleaning agents have to be validated too. In addition, in the case of product changeover, all the product contact components that cannot efficiently be cleaned have to be replaced. Finally, the steam-in-place (SIP) procedure also has to be validated and a media hold procedure for extended time has to be performed.

The simple replacement of the culture ware by using disposable bioreactors minimises the plant validation efforts and reduces the unit operation times. Higher operational flexibility is offered for process adjustments, e.g. in process design or scale. The process workflow is not fixed as it is in a stainless steel facility.

Design elements dedicated to CIP and SIP procedures can be eliminated. This significantly contributes to cost reduction and safety issues. The overall process security is also improved by the reduction of labour operations, easier manual handling and simplified materials and waste flow. Due to the use of sterile connectors as well as sterile welding technologies, the whole process is performed in a fully closed containment. This eliminates the risk of potential cross contamination and reduces the risk of airborne contamination. Disposable products are often presterilised and need only to be opened and plugged into a supporting container or vessel to be ready for production. The transparency of bag systems also allows checking of the fluid level or the overall culture conditions by visual inspection.

The opportunities in process simplification increase the process robustness and reduce onsite preparation work and the engineering costs involved. The costs are further reduced by the lower capital investment. Not only is the bioreactor technology on its own less cost intensive, but also the utilities, space and laboratory requirements are lower. The elimination of extensive cleaning and cleaning validation procedures as well as the decreased use of water and cleaning solutions contribute to the capital savings. There is no need for steam-in-place (SIP) or autoclaving procedures. The sterilisation of the disposable culture ware is provided by the disposable equipment supplier.

The savings in capital investments also allow small biotech companies to produce their products in-house. CMOs, on the other hand, have the opportunity to produce several products simultaneously without physical segregation due to the closed system approach without any risk of cross contamination. This results in a more efficient use of the facility space. The processes themselves should be easily transferable from one facility to another. Process adjustments or retrofitting can be rapidly implemented.

The improvements in the overall efficacy, especially the time and cost savings, meet the challenges in the development of customised processes in an accelerated

time schedule. Therefore, especially in the pilot plant design, disposable bioreactors are currently broadly used for the development and production of new biopharmaceuticals.

Nevertheless, there are still some challenges in the use of disposable bioreactor systems.

The first is that they are limited in scale. In comparison to the traditional stainless steel tanks with vessel volumes up to 20,000 L, the disposable stirred bag bioreactors reached the 1,000-L scale 2 years ago. Single use bag systems are manufactured from 50 mL to 3,000 L. In terms of a disposable facility concept, the 2,000-L scale can be accommodated by disposable bag systems ranging from 50 L to 2,000 L [27]. Containers larger than 1,000 L, however, are limited in portability and take the risk of bag bursting. They are not yet suited to replace fixed tank systems in a high volume manufacturing plant [28].

Other systems, especially hollow fibre bioreactors, are even more limited in the scale up of the culture space. Only the number of cartridges within one culture ware set can be increased; that means a few litres of culture volume for the largest available system. Despite the high cell density inside, for higher volume products, the target amount can only be achieved by running several systems in parallel.

As already described in Sect. 2.2 this is also the technical objective of the Tsunami Bioreactor with its multilayer design, because the single Wave bag is still limited to 1,000 L bag size corresponding to 500 L working volume. Increasing the bag size in all three dimensions changes the hydrodynamic properties of the induced wave and, therefore, also the energy input and the oxygen transfer rate.

In comparison to the traditional stirred-tank bioreactors, the mixing principle in the different disposable systems is less characterised and cannot be simulated as well as in conventional systems. But the description and simulation of the mixing principle and corresponding mass transfer and energy transfer rates are a necessary prerequisite for the successful scale up.

The potential scale down and the availability of process development equipment is also a critical factor. The comparability of the performance of larger disposable systems to the conventional stainless steel bioreactors of the same scale has been shown, but the smallest scale for the S.U.B. from HyClone is 25 L of working volume, and it is even higher for the Xcellerex system. Both use an impeller, but the dynamics of mixing are different. In terms of deviations of large scale production, the opportunities to use a down scaled device to analyse exactly the potential causes are limited.

The use of plastic systems in general has drawbacks regarding pressure and temperature sensitivity and the potential for puncture [29]. In principle, the bags are characterised by the supplier in respect of transferring leachable substances from the contact surface to the liquid. Nevertheless, this does not prevent additional testing under real process conditions.

Reliance on suppliers for both delivery and flexibility in design and customisation, as well as support in validation issues is strongly needed [30].

Due to the use of different films in the different suppliers' bio-process containers, the growth properties of the cells can also change. Limited process control in comparison

to stainless steel bioreactors influences the clone behaviour. Disposable, non-invasive online measurement is not yet sufficiently developed.

Whereas fixed costs for the bioreactor investment and the overall validation efforts are decreased, there are additional costs for the consumables (bags, tubes, filters) and for the disposal of the disposables, especially in commercial production.

For the commercial facility design activities, there is actually no information available regarding the utilisation rate and how many processes fail from an annual perspective. However, the robustness of disposable based processes has to be carefully evaluated. To contribute to the main GMP principles, the supplier diversity also needs to be strengthened.

4 Requirements for the Use of Disposables

From the regulatory perspective, the biopharmaceutical production process should be characterised by deep understanding of the impact of technologies, materials and process conditions on the product quality.

Disposable equipment can influence the drug substance quality in terms of purity, safety and stability. The release of leachables and the integrity of the sterile barrier, as well as the overall system robustness, can contribute to these issues. Necessary steps for customers to evaluate and reduce any potential risks by using disposables are gathering any available supplier information, implementing a risk assessment procedure and, finally, analytical process testing.

4.1 Extractables and Leachables

Disposable bag systems consist of several polymer layers. Ethylene-vinyl-acetate (EVA), ethylene-vinyl-alcohol (EVOH) and polyethylene (PE) are commonly used among others. EVA is a flexible, tough material without plasticisers, but it allows gas and moisture exchange. Compared to EVA, PEs have lower extractable/leachable levels and are inert to a broader range of chemicals. They are often used as the fluid contacting inner film. EVOH is a material with a high gas barrier. The combination of different film materials and the corresponding three-dimensional geometry finally ensures flexibility, resistance and product integrity as well as low product absorbance. Figure 7 shows a film cross section of HyClones BioProcess Container (BPC) systems [19].

Chemical compounds from the culture ware (e.g. bags, hollow fibres, roller bottles) may leach into the solution. Potential leachable and extractable substances are degradation products of the polymer material due to the sterilisation by gamma irradiation and also chemical additives (e.g. anti-oxidants, heat stabilisers, plasticisers). As well as the culture container itself, the plastic tubing is also a potential source and has to be carefully evaluated [31].

Whereas leachables migrate spontaneously from the equipment under recommended conditions of use and storage, extractables are extracted under exaggerated temperature and time conditions [32].

0.8	0.9	1.0	0.9	10.4

Fig. 7 Cross section through the different bag layers, Polyester (*dark grey filled square*), Tie (*light grey filled squares*), EVOH (*open square*), PE (*filled square*)

The potential risks associated with these compounds are toxicity, immunogenicity (acting as adjuvants), product impurities and direct interaction with the drug by affecting its structure, activity or stability.

In general, extensive assessment of potential extractables/leachables is carried out by the vendor of medical devices (e.g. bioreactors) and provided as part of the validation documentation. The test standards to show biocompatibility are prescribed in the United States Pharmacopeia (USP), European Pharmacopeia (EP), Japanese Pharmacopeia (JP) and International Organisation of Standardisation (ISO).

All plastic materials are initially tested for cytotoxicity. Biological reactivity tests, in vitro, are based on elution methods (USP <87>, ISO 10993-5) or the agar diffusion4 test (USP <87>). Several mammalian cells can be used to test for cell lysis or growth inhibition. The material is extracted with Minimum Essential Medium (MEM).

Biological reactivity tests, in vivo, are done according to USP <88> Class VI Biological Reactivity. The test series includes systemic toxicity, intra-cutaneous reactivity and implantation. Similar test methods are also prescribed in ISO 10993-11, -10 and -6.

In addition to these general tests to show biocompatibility, methods with suitable sensitivity and selectivity are needed to characterise and quantify the extractables themselves. Based on risk assessment (patient safety, product interaction) it has to be decided which one will be studied further on as leachables.

Leachables should be monitored during product stability to assess the impact on product quality and to look after the permitted safety limits (ICH Q3C guidelines for the maximum level for residual solvents).

Vendors of disposable systems can support their customers with information about the identified extractables and also the characterised leachables by filling the culture ware with different solutions. However, the final evaluation and risk assessment can only be done under the contact conditions which are specific to the real application. An intensive collaboration and data exchange between the manufacturer of the drug product and the plastic material supplier is strongly recommended [33].

4.2 Integrity and Robustness

The quality of stored contents depends primarily on the characteristics of the plastics around them (e.g. films). In addition to the risk of direct interaction of leachables with the product affecting its quality, the integrity and robustness of the whole system has to be ensured.

Integrity testing is typically done by the vendor as a main part of the container closure validation. It includes a helium leak test, pressure test, dye ingress and microbial ingress challenges [34].

By using hollow fibre AcuSyst bioreactors, the integrity testing is also part of the system set up procedure before starting the fermentation. The system is prepared for operation by a fill and flush procedure to remove leachables and also by a pressure test for leak tightness.

Several parameters are tested for the evaluation of the overall system robustness regarding mechanical load and permeability [35].

The tensile properties describe the material strength and ductility. The test procedures can be found in the American Society for Testing and Materials (ASTM) standard D882. In principle, the material is mechanically stressed to fracture or to be permanently deformed and the strength or percentage of elongation is measured.

The puncture resistance and puncture strength measures the energy that can be absorbed by the material to resist damage or inhibit deformation. The ability to absorb energy over a temperature range can be measured as glass transition temperature (ASTM E1640).

The mechanical forces induced by the fluid movement inside also have to be considered, as well as the energy coming from outside. An appropriate test procedure for the transportability of containers filled with solutions is prescribed in the International Safe Transit Association (ISTA) procedure 2B.

The permeability of plastic materials plays a crucial role in product quality and stability. The gas and water permeability especially can change parameters such as pH, concentration and oxidation potential. Oxygen and carbon dioxide permeability are measured according to ASTM D3985-95, and the testing of water vapour transmission is prescribed in ASTM F1249-01.

The test procedures listed above are only a representative selection to demonstrate that the development of disposables and the understanding of potential risks associated with the handling of plastic materials have reached a high level. This is not mainly driven by the use of disposable bioreactors, but rather by the overall increasing tendency for the application of plastics and also single use materials.

Nevertheless, the development of disposables in the biopharmaceutical manufacturing area can take advantage of the already existing knowledge and apply methods and standards to their specific purpose. Most of the information required can be provided by the supplier of disposable material and equipment, but to assess the risks in a specific application, the conditions specified in the individual process are needed.

5 Current Applications to Production

5.1 Cell Banking

The main objective of cell cultivation for cryopreservation is to keep the cells at a high viability level and to reduce the number of passages to obtain the required quantities.

Depending on the stage of the development process, the number of vials and the level of safety characterisation of the cells usually differ. A well characterised cell bank (Master Cell Bank or Working Cell Bank) is needed as a starting point, especially for GMP production processes. Certainly, the cell bank itself also has to be produced in a safe and robust cultivation and cryopreservation process.

Disposable devices, e.g. T-flasks, roller bottles or culture bags, are well suited and widely used for cell banking purposes. The main advantages are safety and flexibility, as it has already been mentioned. Due to the single use, potential cleaning and cleaning validation efforts can be omitted. Otherwise the production costs of cell banks would be multiplied.

The cell culture space has to be adapted in a flexible manner depending on the number of vials required. Using disposable culture ware, the scale up can be realised by multiplying the number of culture systems, e.g. roller bottles or multi-tray bioreactors. The simplification of the process and the reduction of maintenance and validation work lead to reduced unit operation times, despite the higher efforts in manual operation.

Nevertheless, the scale up concept by increasing the system volume can also be applied. For example, the Wave bioreactor technology offers the operation of different scales from 2 to 50 L bag size with the same rocking platform. Thus, the whole growing process can be covered by one bioreactor system. Not only the handling procedures, but also space and laboratory requirements can be lowered.

It is recommended to adapt the cells to higher cell densities, especially for hollow fibre bioreactor processes. By using the CeLLine bioreactor system, cell densities > 1E + 07 cells mL^{-1} can be reached. The cells can be directly filled into the vials without further centrifugation.

5.2   First Material Production

The IMP (Investigational Medicinal Product) development schedule of recombinant glycoproteins contains cell line and process development, as well as preclinical and clinical production (Fig. 8). The critical parameters for the success of the
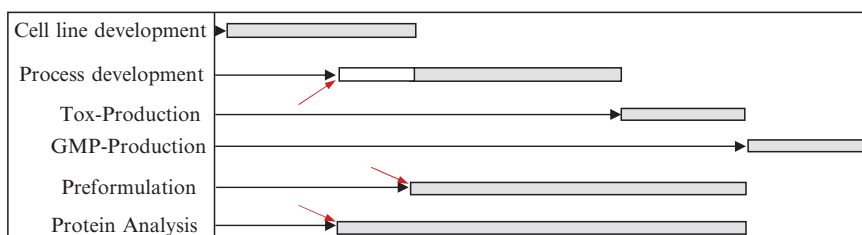
Fig. 8 IMP development. The program steps with a need for first material are marked with *arrows*. The start of the early downstream development (*white block*) can overlap with late stage clone selection procedures. The whole program takes at least 18 months

development program are product quality and process yield, but also the time to clinic.

To save time, different activities should run in parallel, e.g. the final clone selection can overlap with the purification development, or process development with assay qualification. To improve quality, the integration of extended glycoprotein characterisation into cell line and process development is recommended. Glycosylation analysis, in addition to the selection of high yielding cell clones, can avoid the need for larger dosage forms as a result of poor product quality. Suitable activity assays, already established at the beginning of process development, not only provide confidence in the developed process but also lower the risk of lack of capacity and time loss.

However, all these activities assume the availability of at least partly purified product at a very early stage of development. The time to get this material determines the starting point for assay, process and formulation development on the one hand, but also for preclinical proof of concept studies in animal models on the other hand.

The use of disposable bioreactors offers a time and cost efficient way to produce first amounts of the desired product, or even several products, in parallel, e.g. glycosylation variants from different clones or process strategies. A closed containment concept by using sterile welding techniques avoids the risk of cross contamination between the different processes.

Small biotech companies particularly can take advantage of producing sufficient amounts of their products in-house without the engineering background needed for conventional bioreactors. Large pharmaceutical companies with huge screening efforts for a large number of new drug candidates can also design and realise their development strategies more efficiently.

5.3   GMP Production

Disposable bioreactors are suitable for any production purpose. Depending on the required production amount and the stage of development, the different technologies are limited at a certain level in terms of scale and cost effectiveness. Nevertheless, several technologies, e.g. wave bioreactors or disposable stirred bag bioreactors, are capable of supporting drug development up to late stage clinical development and even market supply for high potency and low volume products. Cytokines with low therapeutic doses in the mg range can be produced in quantities of at least 100 g per year by using one 1,000-L disposable bioreactor, even if the calculation is based on a low final expression level of 10 μg mL^{-1} . Depending on the therapeutic concept and structural design, antibodies can also be administered in therapeutic doses below 1 mg [36]. Proteins with an annual market demand of up to 1 kg are very attractive in terms of price per gram (compare with Fig. 1). The development of biosimilars and second generation products

will further split up the market for each individual protein and, therefore, decrease the market demand. The use of disposable bioreactors can significantly contribute to the highly needed time reduction in development and cost reduction in production for these products.

For high volume products, disposable bioreactors can be used for preclinical and early clinical material production or as a seed train for the larger, stainless steel production vessel. Starting the clinical development with smaller disposable devices and continuing with the traditional stainless steel technology bears the risk of changes in the product quality due to slight changes in the production process. However, changes in the production process due to improvements in yield or the scale up procedure itself and the consequential comparability studies are quite normal and many case studies exist even after market entry of various products [37].

However, the potential risks can be minimised by a deep understanding of the influence of process parameters on product quality. This approach is supported by the FDA's Process Analytical Technology (PAT) initiative.

On the other hand, the use of disposable technologies with similar scale up concepts or even equal mixing principles can enable a seamless tech transfer to large-scale, stainless steel bioreactor facilities.

In the case of exceeded capacities, even for clinical material supply, disposable bioreactors are valuable as seed trains for the large production vessel. The higher operational flexibility in combination with the lack of hard piping requirements and the reduction of fixed installation as well as validation and cleaning costs have a significant impact on production.

In a case study published recently, the production process was designed to use a Wave bioreactor sequence of 20, 50, 200 and 1,000 L and finally inoculate the 2,000-L stirred-tank bioreactor for production. The scale up could be successfully realised and the entire manufacturing process was streamlined [38].

The fact that disposable bioreactors are already in use at a final production scale, even for commercial production, is underlined by the following two case studies.

5.4   Case Study 1: Production of Erythropoietin

The first recombinant erythropoietin product from Amgen Inc. was approved by the FDA in 1989. Erythropoietin acts as a hormone and stimulates the formation of red blood cells (erythropoiesis) in the bone marrow. Consequentially, the drug is used for the treatment of anaemia induced by loss of blood, kidney impairment or toxicity of chemotherapeutic drugs.

The first amounts of human EPO were purified from urine in 1977 [39]. Almost 10 years later, the recombinant expression in CHO cells was reported [40]. Due to the high content of carbohydrates (approximately 40%), mammalian cells are essential to produce erythropoietin. The carbohydrates influence both the activity and stability of the drug. The level of terminal sialic acids is a critical

parameter. While a lower level of sialic acids increases the affinity of EPO for its receptor, with a consequent increase in its in vitro bioactivity, at the same time the hepatic clearance is increased, with consequent reduction in its in vivo bioactivity [41]. As well as the cell substrate, the production process also impacts the glycosylation profile. The first commercial production process of recombinant erythropoietin is based on the cultivation of CHO cells in a large number of roller bottles [42]. Compared to the standards today, this manufacturing process seems to be antiquated, but it allowed Amgen to produce sufficient quantities of clinical material in a short time. The roller bottle technology is still in use for the production of the erythropoietin products Epogen/Procrit and Eprex and the second generation product Darbopoietin alfa with two additional glycosylation sites.

More modern bioreactor-based methods are involved in the development of erythropoietin biosimilars. However, the selection of the production technology depends on the overall development time, the expected cost of goods, the market demand and the regulatory hurdles to show similarity. The influence of the production process on glycosylation was shown by the comparison of erythropoietin produced in roller bottles and in a hollow fibre bioreactor system [41]. The majority of the roller bottle preparations contained more than 40% (mol/mol) tetrasialylated tetra-antennary N-glycans, whereas the protein isolated from the hollow fibre process contained less than 30 mol% of these structures. The content of tetrasialylated tetra-antennary structures was identified to contribute significantly to the in vivo biological activity of erythropoietin.

5.5   Case Study 2: Production of Capromab Pendetide

Capromab pendetide (Prostascint®) from Cytogen Corp. is indicated as a diagnostic imaging agent in newly-diagnosed patients with biopsy-proven prostate cancer. The drug consists of the murine IgG1 monoclonal antibody capromab, chemically conjugated to a linker-chelator peptide group. The conjugate finally reacts with Indium In111 to form a radioimmune complex [43]. The product was approved by the FDA in 1996 and represents the first injectable biopharmaceutical protein produced in a hollow fibre bioreactor system.

The Summary Basis of Approval (SBA) lists three different processes for the production of the monoclonal antibody. The description of the original manufacturing process was censored by the FDA. The subsequent two processes B and C were also heavily redacted, giving no information about the bioreactor system in process B. Only process C is employed for commercial production and specified by the use of an AcuSyst-XCell hollow fibre bioreactor with a culture ware set containing six hollow fibre cartridges. For the procedure described in the SBA, two sequential harvests were collected and purified separately to show consistency and stability in perfusion.

Due to the diagnostic application, the single dose vial contains only 0.5 mg. The use of the hollow fibre bioreactor system should allow the production of at least tens of thousands of doses per run.

6   Disposable Bioreactor Based Facility Design

The design of a new facility is mainly conditioned by the parameters purpose, capacity, speed, costs, safety, regulatory acceptance and flexibility.

Regarding the life cycle of new biopharmaceutical drugs, it can be distinguished between pilot and commercial plant activities. Whereas the first one covers cell line and process development as well as the production of preclinical and clinical material, the large-scale facilities are intended to be used for the late clinical and market material supply (Fig. 9).

6.1   The Pilot Plant Concept

The pilot plant design depends on the number of drugs to be developed within a certain time frame or simultaneously, the planned production mode and the expected capacity to supply the required protein amounts for preclinical and clinical testing purposes. The major drivers are flexibility and speed. The time to enter the clinical trials is a key success factor particularly for the overall drug development program. The use of disposable equipment, particularly bioreactors, complies perfectly with these requirements.

The high level of flexibility by using disposables is mainly ensured by short change over procedures and the use of portable equipment with low space and cleaning validation requirements. The lack of fixed piping eliminates the necessity for centralised steam and WFI production.

The broad variety of disposable bioreactor technologies using different mixing principles allows the development of cell and product tailored production processes. The process does not have to fit to only one existing technology. Due to the low capital investment, the low space requirements combined with the portability of the
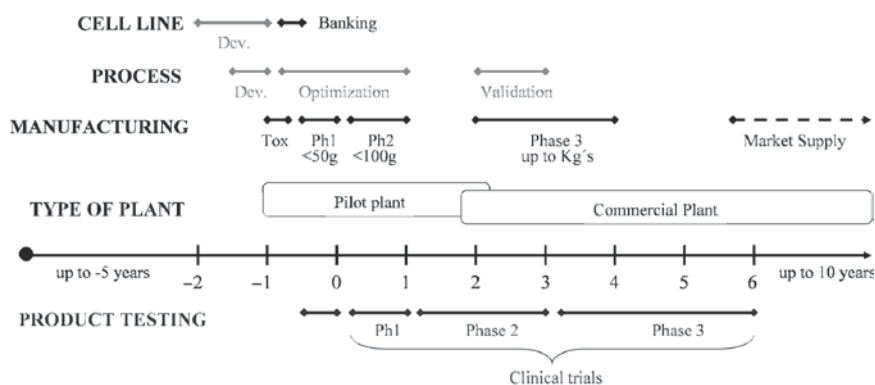
Fig. 9 Life cycle of complex proteins [44]

systems, different technologies and production modes can be applied during the development without changing the overall pilot plant concept.

Process adjustments as an outcome of improvements during production can also be rapidly implemented. Finally, the whole process should be easily transferable from one site to another, thus minimizing the risk of substantial time loss.

Other time savings offered by the use of disposable bioreactors are the fast change over procedures due to a simple replacement of the culture ware set instead of extensive CIP and SIP processes. This also reduces the facility validation efforts.

The use of sterile connectors as well as sterile welding technologies enables the production in fully closed containment. Thus, several products or product candidates can be developed and manufactured in the same suite, being physically segregated only by use of closed systems. The risks of cross contamination due to insufficient cleaning or airborne contamination are eliminated.

Despite the flexibility to integrate and operate different technologies and scales in the pilot plant, the overall scalability is still limited in comparison to stainless steel bioreactors. To assure the supply of material up to the requirements of clinical phase II studies, the production capacities in a pilot plant should be extended by the capabilities to develop high-expression cell lines. The combination of clone selection with media optimisation and early process development in small disposable bioreactor devices, e.g. shaker tubes, allows high throughput screening with a high susceptibility to find the right clone that is suitable for production.

To support cell line and process development, the pilot plant activities should also include extensive glycoprotein characterisation, e.g. glycosylation analysis or bioactivity testing. Strategies to modify glycosylation profiles and to decide on clones and process design by considering both product quantity and quality can avoid the need of larger dosage forms.

A deep understanding of how process parameters can influence the protein structure and activity should also give the supportive information needed or even deviation explanation during scale up or process changes on a commercial scale.

6.2 The Commercial Facility Design

Biopharmaceuticals are the fastest growing segment in the life science market. The growth rates are double-digit and are also expected to outgrow traditional Pharmas in the future. Biopharmaceuticals can be produced in-house or externally by contract manufacturing organisations (CMOs). The CMO market was $ 2.1 billion in 2006 and is expected to have CAGR (capacity annual growth rate) of 15% until 2011 [45]. In line with this dynamic growth, a significant increase of mammalian cell based manufacturing capacities is needed within the next years to meet the demand.

A trend in the market has been identified: a shift from large-scale, single product, multi-ton facilities towards flexible, cost effective, medium size multi-product facilities. The graph in Fig. 10 illustrates the trend from large-scale to medium-scale facilities.
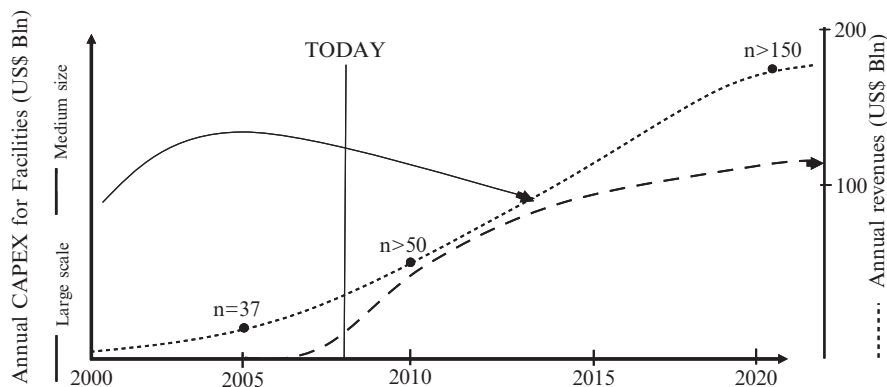
Fig. 10 Global trends for commercial mammalian cell based facility investments over the next decades with expected growth of the number of marketed mammalian cell based biologics (*filled circle*) to >150 in 2020. In early 2000, erection of large scale facilities, equipped with 2–10 trains of 10–20K final stirred tank volume and costs of US$ 100–700 Mio per plant were the major investment activities on the market. The main decision criteria for such plant investments were driven by large capacity, regulatory acceptance and robustness issues. Since last 3–5 years investments in medium scale multi product facilities, designed for annual capacity of round about 50 kg net product and investment costs of less than 100 Mio US$ are increasing. Such facilities seemed to be much more flexible to address dynamic changes within the biologics markets

Increasing expression yields and improvements in the activity, as well as the prolongation of half-life of recombinant drugs, strengthens this tendency. The competition between several biopharmaceuticals going in the same direction and the development of biosimilars and follow-on drugs, are forcing the industry to reduce costs and to be flexible in terms of potential market reduction.

The major drivers in commercial facility design are safety and costs. Over the last 2 years, the pharmaceutical industry has learned to accept the fact that manufacturing facilities built on fully disposable upstream technology (bioreactors) and implementing stepwise disposable downstream techniques (protein purification system) can significantly reduce the CAPEX (capital expenditures). Several studies with individual assumptions were pursued by Stedim and Biopharm Services to evaluate the impact of disposable technologies on investment costs in facility design, and additionally COGs in operation.

The concept study reported in 2005 compared the design of a 1,000-L perfusion process by using either single use systems, e.g. aseptic connection technologies and single use bags, with traditional equipment [46]. The results showed approximately 40% in capital savings. The reduction in the COGs (including capital) in € g^{-1} protein was calculated to be almost 20%. Whereas the overall labour intensiveness could be reduced, the costs for consumables were significantly higher.

An older study reported in 2002 compared a 2,000-L stainless steel bioreactor vessel with single use disposable bag technology of the same size. Approximately 20% of capital savings and almost 10% reduction in the COGs were estimated [47].

The effective capital and operational savings depend finally on the individual concept of the designed facility. Key parameters such as scale, single or multi-product purpose and also the availability of integrated disposable solutions strongly influence the assessment of costs.

Beside the economic assumptions, disposable based facility concepts offer improved process security. Labour operations are reduced by avoiding extensive cleaning and sterilisation procedures. Innovations in aseptic connections allow the removal of media or buffer solutions outside the processing area. Materials and waste flows are simplified.

The work in fully closed and single use containment eliminates the risk of cross contamination and enables concurrent activities. The manufacture of several products in parallel increases the overall plant utilisation. Retrofitting of existing facilities is significantly easier with disposables than with fixed equipment [48]. The modularity of single use processes enables the fast build out of capacities and simplifies potential technology transfers from one facility to another. Both product developing companies and CMOs can take advantage from the cost efficient, flexible and safe manufacturing strategy via disposable facility concepts.

7   Summary and Outlook

New glycoprotein's, second/third generation products and a growing number of biosimilars are continuously entering the biopharmaceutical arena. Exploding R&D costs, increasing regulatory requirements and growing cost pressure in the health care sector have created a significant demand for increasing potency, for optimizing pharmacokinetics and, simultaneously, for cutting manufacturing costs for glycoprotein based pharmaceuticals. Current development in process science (e.g. high expression systems and tools for glycodesign) in combination with upcoming disposable based up- and downstream technologies may resolve this apparent bottleneck. Today, commercially available disposable technologies cover only discrete fragments of the entire glycoprotein processing chain, (see Fig. 11) but huge efforts are underway to close remaining gaps. Different articles in the book emphasise these aspects. A robust supplier industry has been developed for the last few years to provide disposable media containers, harvest bags and bioreactors. The industry has gained substantial information on the mechanical robustness of polymeric materials in pharmaceutical application as well as its biosafety with regard to leachable substances. Validated sterile tube connectors and first reliable non-invasive pH and pO_2 probes have entered the market

Therefore, as well as the use of traditional stirred-tank trains of up to 20,000 L vessel volume, the first disposable based bioreactors appeared in existing mammalian based manufacturing facilities. Since 2002, ProBioGen has been operating one of the first GMP pilot plants worldwide, relying entirely on disposable upstream technology. With the industry wide acceptance of the advantages of disposable based technologies for bio-manufacturing, more and more pilot plants of this type are appearing both in product companies and at CMO sites. This development creates
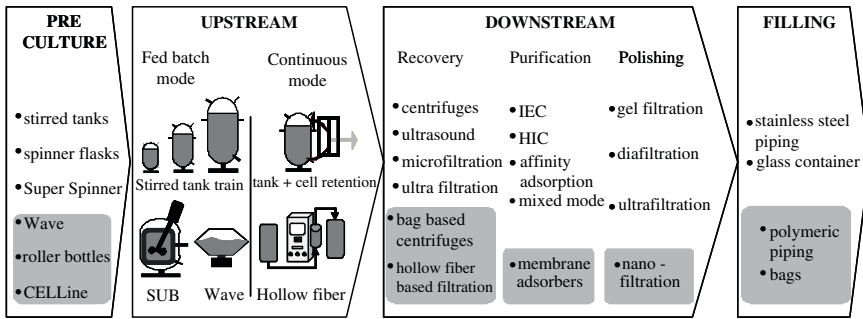
Fig. 11 Current biopharmaceutical glycoprotein processing (*filled squares* disposable systems)

a perfect environment to aggressively develop disposable technologies and plant design to enter commercial manufacturing markets.

To capitalise fully on the economical advantages of disposable technologies for glycoprotein processing, flexible multi-product plants, based on entirely disposable process chains, are envisioned. As shown in Fig. 12, such a facility may ideally consist of:

– Disposable pre-culture equipment operating in classified areas
– Fully disposable closed upstream bioreactor systems operating either in fed batch or continuous perfusion mode in classified areas
– Disposable media and waste holding containers operated in grey areas and sterilely connected to closed bioreactor systems with polymeric tubes through the walls
– Disposable cell recovery and product concentration equipment in classified upstream processing area sterilely connected to a bioreactor harvest line
– Disposable purification equipment (single use filtration units, membrane absorber technology) operated in classified areas
– Disposable pipe and container based bulk filling equipment

Once such entirely disposable based process chains can be combined with an intelligent process suite and facility design, capital investments and costs of goods can be decreased additionally due to the significant reduction of dedicated clean room areas, hygienic regime requirements and work load for process personnel. The design needs to ensure handling of the whole media, waste and downstream buffer flow in grey areas segregated from classified processing areas and flow of process personnel. A schematic concept is given in Fig. 12.

To achieve the goal, research and development need to be focussed on:

– Validated sterile connectors and/or welding technologies, allowing not only frequent connection but also to disconnect polymeric tubes in a sterile way
– Disposable continuous perfusion upstream technologies
– Closed disposable cell recovery and product concentration systems, operable in upstream suites
– Disposable downstream solutions for all required purification and polishing principles
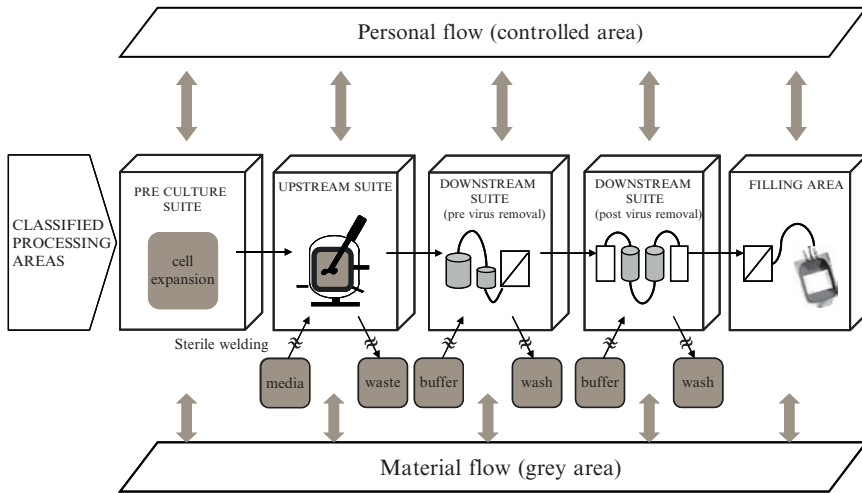
Fig. 12 Flexible multi product manufacturing plants operating fully disposable based process chains are envisioned to serve future biopharmaceutical market needs. Capital and operating costs are reduced by substantial reduction of classified processing areas

– Fully disposable bulk filling lines

Thus our industry is looking at an exciting decade of both implementation of existing and development of additional disposable based biopharmaceutical processing technologies for glycoprotein manufacturing.

References

1. Market study (2007) Biomanufacturing strategies: market drivers, build-vs-buy decisions and opportunities in contract relationship management. Business Insights, September
2. Jain E, Kumar A (2008) Biotechnol Adv 26:46
3. Kanda Y, Yamada T, Mori K, Okazaki A, Inoue M, Kitajima-Miyama K, Kuni-Kamochi R, Nakano R, Yano K, Kakita S, Shitara K, Satoh M (2007) Glycobiology 17:104
4. Mori K, Iida S, Yamane-Ohnuki N, Kanda Y, Kuni-Kamochi R, Nakano R, Imai-Nishiya H, Okazaki A, Shinkawa T, Natsume A, Niwa R, Shitara K, Satoh M (2007) Cytotechnology 55:109
5. Knazek RA, Gullino PM, Kohler PO, Dedrick RL (1972) Science 178:65
6. Eibl R, Eibl D (2007) PROCESS special edition, ACHEMA World Wide News 2:8
7. Terrier B, Courtois D, Hénault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Pétiard V (2007) Biotechnol Bioeng 96:914
8. Kybal J, Sikyta B (1985) Biotechnol Lett 7:467
9. Singh V (1999) Cytotechnology 30:149
10. Marjanovic D, Greller G (2007) Supplement, BioPharm Int 20:38
11. The Wave bioreactor story. www.wavebiotech.com/about_us/about_us.php Accessed on the 11th of July 2008

12. Houtzager E, van der Linden R, de Roo G, Huurman S, Priem P, Sijmons P (2005) BioProcess Int June:3, pp 60–66
13. Tang YJ, Ohashi R, Hamel JF (2007) Biotechnol Prog 23:255
14. Pierce LN, Shabram PW (2004) BioProcess J 4:51
15. Ohashi R, Singh V, Hamel JF Presented at ESACT conference 2001 in Sweden
16. Kadwell SH, Hardwicke PI (2007) Methods Mol Biol 388:247
17. Negrete A, Kotin RM (2007) J Virol Methods 145:155
18. Genzel Y, Olmer RM, Schäfer B, Reichl U (2006) Vaccine 24:6074
19. Wolpers F (2006) Biological Europe, conference presentation, Amsterdam, The Netherlands, 19–22 June 2006
20. Galliher PM (2007) BioProduction, conference presentation, Berlin, Germany October 30–31
21. Chu L, Robinson DK (2001) Curr Opin Biotechnol 12:180
22. Sandig V, Rose T, Winkler K and Brecht R (2005) In: Gellissen G (ed) Production of recombinant proteins: novel microbial and eukaryotic expression. Wiley, Weinheim, p 233
23. Gardner TA, Ko SC, Yang L, Cadwell JJ, Chung LW, Kao C (2001) Biotechniques 30:422
24. Cadwell JJ (2004) Am Biotechnol Lab July:14. vol. 22
25. Langhammer S, Brecht R, Marx U (2007) Genet Eng News 27:34
26. Kranjac D (2004) BioProcess Int 2:86
27. Monge M, Sinclair A (2005) In: Godia F, Fussenegger M (eds) Animal cell technology meets genomics: Proceedings of the 18th ESACT Meeting Granada, Spain, May 11–14, 2003. Springer, Netherlands, p 667
28. Kundu S (2007) Future Pharmaceuticals, Q4. vol. 100
29. Fox S (2005) Contract Pharma June:62. vol. 7
30. Wrankmore M (2005) Biologicals Manufacturing Summit, conference presentation, London, UK, February 23–24
31. Jenke DR, Story J, Lalani R (2006) Int J Pharm 315:75
32. Markovic I (2007) AAPS National Biotechnology Conference. San Diego, June 24–27, 2007
33. Jenke D (2007) PDA J Pharm Sci Technol 61:17
34. Vega H, Schultz TJ (2007) Future Pharmaceuticals Q3 2007:96
35. HyClone (2004) Technical papers series: Disposable Flexible Container Systems for Cell Culture Media and Other Sterile Liquids'; Revision 2, August 2004; Compiled and Edited by the HyClone Technical Staff
36. Press Release Fresenius, 22.03.2007, www.fresenius.de/internet/fag/com/faginpub.nsf/Content/Press+Releases+2007 Accessed on the 11th of July 2008
37. Harris R (2006) Comparability for Biologics, conference presentation, Zürich, Switzerland, June 27–28
38. Charles I, Lee J, Dasarathy Y (2007) BioPharm Int Suppl Nov. 2:31. vol. 20
39. Miyake T, Kung CK, Goldwasser E (1977) J Biol Chem 252:5558
40. Lin FK, Suggs S, Lin CH, Browne JK, Smalling R, Egrie JC, Chen KK, Fox GM, Martin F, Stabinsky Z (1985) Proc Natl Acad Sci U S A 82:7580
41. Yuen CT, Storring PL, Tiplady RJ, Izquierdo M, Wait R, Gee CK, Gerson P, Lloyd P, Cremata JA (2003) Br J Haematol 121:511
42. Rader RA (2005) Biopharmaceutical products in the U.S. and European Markets, 4th edn. Bioplan Associates, Rockville, USA, p 121
43. Rader RA (2005) Biopharmaceutical products in the U.S. and European Markets, 4th edn. Bioplan Associates, Rockville, USA, p 337
44. Brecht R, Koch S, Riedel M, Sandig V, Marx U (2005) BioPharm Int July:22. vol. 18
45. High Tech Business Decisions (2007): Biopharmaceutical Contract Manufacturing 2007: Quality, Capacity and Emerging Technologies'; Industry Report
46. Sinclair A, Monge M (2005) Biopharm Int, Supplement, October:26.vol. 18
47. Sinclair A, Monge M (2002) Pharma Eng 22:20
48. Terryberry JW, Thor G (2006) Biodisposables utility and technological advances. D&MDNew York, USA

Use of Orbital Shaken Disposable Bioreactors for Mammalian Cell Cultures from the Milliliter-Scale to the 1,000-Liter Scale

Xiaowei Zhang, Matthieu Stettler, Dario De Sanctis, Marco Perrone, Nicola Parolini, Marco Discacciati, Maria De Jesus, David Hacker, Alfio Quarteroni, and Florian Wurm

Abstract Driven by the commercial success of recombinant biopharmaceuticals, there is an increasing demand for novel mammalian cell culture bioreactor systems for the rapid production of biologicals that require mammalian protein processing. Recently, orbitally shaken bioreactors at scales from 50 mL to 1,000 L have been explored for the cultivation of mammalian cells and are considered to be attractive alternatives to conventional stirred-tank bioreactors because of increased flexibility and reduced costs. Adequate oxygen transfer capacity was maintained during the scale-up, and strategies to increase further oxygen transfer rates (OTR) were explored, while maintaining favorable mixing parameters and low-stress conditions for sensitive lipid membrane-enclosed cells. Investigations from process development to the engineering properties of shaken bioreactors are underway, but the feasibility of establishing a robust, standardized, and transferable technical platform for mammalian cell culture based on orbital shaking and disposable materials has been established with further optimizations and studies ongoing.

Keywords Disposable bioreactor, Mammalian cell culture, Orbital shaking, Oxygen transfer rate, Scale-up, Shaken bioreactor

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Contents

Abbreviations

CFD   Computational fluid dynamics
CHO   Chinese hamster ovary
DO   Dissolved oxygen
HEK   Human embryonic kidney
OTR   Oxygen transfer rate
PCV   Packed cell volume
SIP   sterilization in place

1   Introduction

Due to their capacity for proper protein folding, assembly, and post-translational modification, cultivated mammalian cells have become the dominant host for the production of recombinant proteins for clinical applications, contributing up to 50% of all recombinant protein pharmaceuticals produced [1]. The enormous commercial success of therapeutic proteins along with advances in genomics has enhanced the number of protein drug candidates awaiting investigation with hundreds of proteins now in clinical trials. As a consequence, there is an increasing demand for simple to operate and cost-effective bioreactor systems for the cultivation of mammalian cells at volumetric scales from a few milliliters to 1,000 L for process development and the rapid production of gram amounts of recombinant protein for in vitro testing and proof-of-concept preclinical trials.

Stirred-tank bioreactors are the dominant mammalian cell culture system for scales beyond a few liters. However, the high cost of stirred-tank bioreactors limits their availability. In comparison, spinner flasks are much less expensive, but their application is restricted by poor oxygen transfer capacity, and so they are commonly used at scales of less than 1 L.

Orbitally shaken vessels, especially "Erlenmeyer" flasks[1] with nominal volumes up to 5 L, are widely used for the cultivation of microbial hosts because they are easy to handle and inexpensive. Continuous efforts in bioprocess engineering over the past decade have made them attractive alternatives to conventional stirred-tank bioreactors [2]. More recently, orbital shaking technology has been reported for the cultivation of mammalian cells in cylindrical or square-shaped vessels [3–7] and within disposable bags [8] with working volumes typically in the range of 5 mL to 30 L. Cultivations were also performed successfully in our lab using 200-L and 2,000-L single-use bags with working volumes of 50% of the nominal volume. In contrast to stirred-tank bioreactors in which an invasive agitator is used for mixing, the vessels were agitated on an orbital shaker. This mixing strategy makes it very convenient to employ disposable materials – a recent trend in biopharmaceutical industry – as culture vessels [9], benefiting bioprocesses with improved flexibility and reduced costs compared to conventional stainless steel systems.

To establish a workable technology platform, investigations of the basic engineering properties of shaken bioreactors are underway, and the first results are presented in this chapter. The volumetric mass transfer coefficient ($k_L a$) values of shaken bioreactors from the milliliter scale to the 1000-L scale were determined, and the operating parameters having an impact on oxygen transfer were investigated. The results led to further improvements in reactor design aimed at increasing oxygen transfer rates (OTR). Also, the complex two-phase free-surface flows developed within these vessels have been analyzed using an advanced computational fluid dynamics (CFD) model based on resolution of the Navier–Stokes equation. The free-surface shapes and the velocity fields obtained in the simulations compared well with experimental data. Insights obtained by these studies have led to the primary description of the engineering profile of shaken bioreactors, a step needed for efficient scale-up and further process optimizations.

2 Small-Scale Shake Technologies for Animal Cell Cultures

In animal cell technology, the availability of an appropriate small-scale or scale-down system is essential for the understanding and development of new cell culture processes. Recently, as an alternative to existing systems such as shake flasks and

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[1] **Richard August Carl Emil Erlenmeyer** (28 June 1825–22 January 1909): He studied at Gießen under Justus von Liebig and at Heidelberg under Friedrich Kekulé. He also associated himself with Robert Bunsen in the study of fertilizers. Erlenmeyer was professor of chemistry at the Munich Polytechnic School from 1868 to 1883. His experimental work included the discovery and synthesis of several organic compounds, e.g., isobutyric acid (1865); in 1861 he invented the conical flask that bears his name. Among the first to adopt structural formulas based on valence, he proposed the modern naphthalene formula of two benzene rings sharing two carbon atoms.
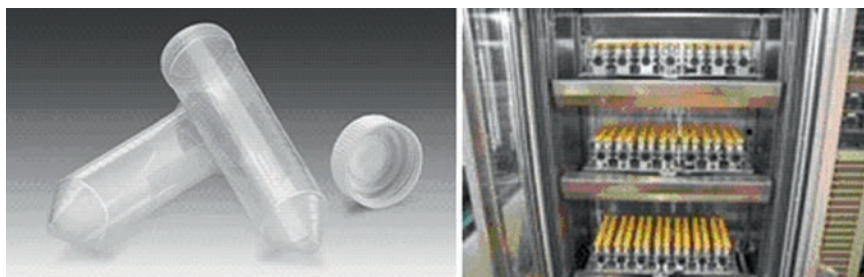
Fig. 1 The 50-mL shake tubes with filter cap (CultiFlask 50) (*left panel*) and their application as parallel bioreactors in a CO_2-incubator with humidity control (*right panel*). Typically the tubes were orbitally shaken at 180–220 rpm and a shaking diameter of 50 mm

agitated multi-well plates [10], modified 50-mL ventilated centrifugation tubes[2] (termed TubeSpin®-bioreactors) have been tested [4] (Fig. 1). The tubes, which were developed at the Swiss Federal Institute of Technology in collaboration with the company ExcellGene SA, are fitted with a ventilated cap allowing passive gas exchange while preventing contamination (Fig. 1a). Using an adequate incubator and orbital shaker, several hundred shake tubes can be operated simultaneously [11] (Fig. 1b). Previous studies have partially defined the physico-chemical characteristics of these disposable 50-mL shake tubes [4, 11]. Parameters such as working volume, water evaporation, and oxygen supply have been studied and optimized.

Apart from the ease-of-use and the potential for high-throughput cell line development, the 50-mL TubeSpin® system was developed because of its bioreactor-like behavior [4]. Under moderate orbital shaking speeds, these tubes have a sufficient oxygen transfer capacity, a low shear, and a defined gas–liquid interface surface which make the tube an ideal bioreactor system for small-scale studies and other high-throughput applications. With working volumes of 5–25 mL, samples of a few microliters can be taken daily for monitoring purposes. The system fills a gap between very small-scale shake systems used for true screening approaches (multi-well plates) that lack bioreactor-like performance for suspension cultures and larger systems such as lab- and pilot-scale bioreactors for process development.

As an example of the utility of the tubes, a multi-parameter screening for cell growth and transient gene expression is shown here. CHO DG44 cells originating from a single pool were first transfected in different chemically defined media using an optimized polyethylenimine-based protocol in 1-L shake bottles [12]. A monoclonal IgG1 antibody was expressed as a reporter protein. The transfected cells were then cultivated overnight and distributed into multiple TubeSpin®-bioreactors the next day. Then peptones from soy origin (coded P01 to P06) were added and the temperature was reduced to 31 °C. On day 6, antibody concentration was assessed by sandwich ELISA and biomass was determined by the packed cell volume (PCV) method [13] (Fig. 2). Each medium and peptone combination was repeated in three individual tubes.

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[2] The 50-mL shake tubes with filter cap originally sold under the name TubeSpin®-bioreactors (Techno Plastic Products AG, Trasadingen, Switzerland) are also available under the tradename CultiFlask 50 Tube by Sartorius Stedim AG (Göttingen, Germany). A second generation CultiFlask 50 tube with a modified geometry is expected to be released soon.
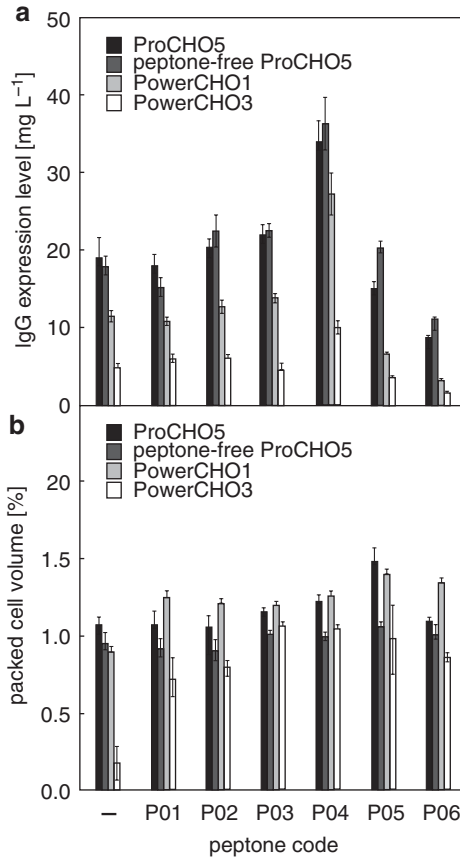
Fig. 2a,b Antibody concentrations (**a**) and packed cell volume (**b**) as a function of different peptones added to the media at a concentration of 0.5% (w/v). The cells were transfected in four different chemically defined media as indicated at day 0 and distributed into 50-mL shake tubes 24 h later. At day 1, the peptones were added and the temperature was shifted down to 31 °C. Average day 6 values of different 50-mL shake tubes are reported ($n = 3$)

This example of optimization using TubeSpin®-bioreactors stressed the importance of relying on an appropriate scalable technology. Here, combinations of media and peptones that significantly increased the antibody expression level (peptone P04 in Fig. 2) were identified with a high degree of confidence and confirmed previous findings of others [14, 15]. However, it should be noted that the validity of such a screening is dependent upon the cell line, the transfection method, the recombinant protein, and the process. Peptones that positively influence one process may have inhibitory effects in another. Variability in raw materials and in the peptide hydrolysis process may also affect the results. Therefore, each new optimization should be considered independently. The use of an adequate small-scale screening technology is therefore critical. Since each 50-mL tube reproduces the same physico-chemical growth conditions, cell line and medium optimization is facilitated when using multiple tubes in parallel. Thus, optimal conditions can be assessed with a high degree of confidence.

3   Optimal Oxygen Transfer Properties
      in Shake Cultivation Systems

The high cell densities repeatedly observed in the 50-mL tubes indicate that oxygen is transferred to the liquid phase more rapidly than it is taken up by the cells. To confirm this assumption, a systematic characterization of oxygen transfer was performed. The oxygen transfer rate (OTR) per unit of bioreactor volume is given in (1) [16].

$$OTR = k_L a(C_L^* - C_L) = k_L a(Lp_G - C_L) \tag{1}$$

where k_L [m h^{-1}] is the liquid mass transfer coefficient, a [m^{-1}] the specific interface area (gas to liquid phase) of mass transfer per liquid volume, C^*_L [mg L^{-1}] the oxygen concentration at saturation, C_L [mg L^{-1}] the measured dissolved oxygen concentration, L [mg L^{-1} bar^{-1}] the solubility of oxygen in the liquid phase, and p_G [bar] the partial pressure of oxygen in the gas phase.

The specific liquid mass transfer coefficient $(k_L a)$ is often used to compare the efficiency of different bioreactors in terms of oxygen transfer both for microbial and animal cell bioprocesses [17, 18]. The usual range of $k_L a$ values in microbial cultivation systems is 100–400 h^{-1}, and these values are typically achieved through very high revolutions of an impeller in addition to the sparging of oxygen into the vessel from below the impeller [17]. For animal cell cultivation, due to lower oxygen uptake rates compared to microbial hosts, the required $k_L a$ values are more in a range between 1 and 25 h^{-1}. According to (1), an increased oxygen transfer results when either k_L or a is increased. In shake cultivation systems such as 50-mL tubes, normally the $k_L a$ increases with the agitation speed.

Numerous studies recently evaluated the $k_L a$ in small-scale shake cultivation systems such as micro-well plates [19–21] and shake flasks [22–24]. In these studies, the monitoring of dissolved oxygen (DO) was performed using novel optical sensors. The measurement principle is based on the quenching of luminescence caused by collision between molecular oxygen and luminescent dye molecules in the excited state. The system uses an immobilized fluorescence sensor made of two fluorophores. The first has a signal intensity that is related to the dissolved oxygen while the second is insensitive to oxygen and is used as a reference. Both fluorophores are excited at the same wavelength but emit light at two distinct wavelengths [25]. Turbidity and changes in the shaking rate have no influence on the measurement. Also, there is no cross sensitivity for CO_2 and other compounds or for pH variations. The non-invasive optical sensing of DO proved to be well suited for shaken bioreactor systems and is relatively easy to adapt to different vessel geometries and sizes.

3.1   Oxygen Transfer in 50-mL Shake Tubes

Oxygen sensing spots were immobilized on the inner wall of 50-mL Tubespin®-bioreactors (Fig. 3). A polymer optical fiber was used to transmit the light signal
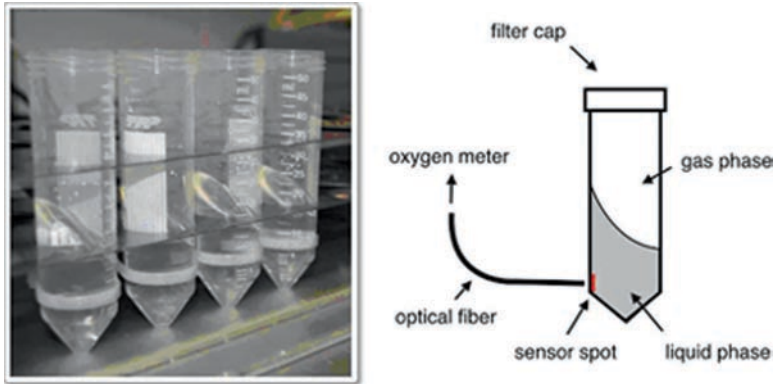
Fig. 3 Some 50-mL shake tubes with 20 mL water were agitated at 200 rpm (*left*). Schematic diagram of the optical oxygen sensor setup (*right*). The sensor spot was placed right above the conical bottom part of the 50-mL shake tube. The diameter of the sensor spot was 3 mm

between the OXY-4 module and the sensor spot (PreSens GmbH, Regensburg, Germany). According to the manufacturer, the response time of the oxygen sensor in stirred systems is very brief (less than 30 s) and was neglected in this work. Due to their small size, the sensor spots did not interfere with the fluid dynamics in the tubes. The oxygen transfer measurements were carried out using a lab shaker fitted with a tube holder specifically designed for multiple 50-mL Tubespin®-bioreactors (Adolf Kühner AG, Birsfelden, Switzerland). The shaker diameter was set at 50 mm. The oxygen monitoring system was calibrated by saturating the liquid phase with either air or N_2. The classic dynamic method [26] was used for the evaluation of the $k_L a$. The liquid phase was saturated with N_2 to reach a constant value of the DO near zero. After N_2 addition was completed, the gas phase was rapidly replaced by air. Immediately after this, a given agitation speed was applied. The resulting DO increase was monitored until saturation was reached. The $k_L a$ was calculated from the slope of the mass balance (2)

$$\ln \frac{(C^* - C_1)}{(C^* - C_1)} = k_L a(t_2 - t_1),$$ (2)

where C^* [mg L^{-1}] is the saturation DO[3], C_1 and C_2 [mg L^{-1}] are the DO at time t_1 and t_2 [h] respectively.

To characterize the oxygen transfer effects in 50-mL Tubespin®-bioreactors, the $k_L a$ was assessed for different combinations of agitation speed and working volume. The experiments were performed with pure water at 37 °C without a filter cap. The $k_L a$ was noticeably affected by the variation of the agitation speed (140–280 rpm). Values varied between 7 and 45 h^{-1} for a working volume of 20 mL and between 3 and 22 h^{-1} for a working volume of 30 mL (Fig. 4a). It was assumed that the $k_L a$ varied

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[3]The DO was 6.72 mg L^{-1} at 37°C, assuming normal pressure conditions (1,013 mbar).
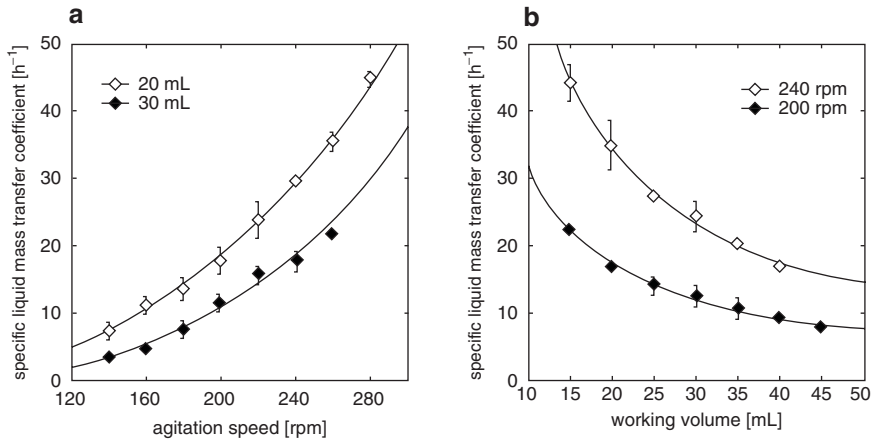
Fig. 4a,b Experimentally estimated specific liquid mass transfer coefficient $(k_L a)$ as a function of agitation speed with constant working volume (20 and 30 mL) (**a**) and as a function of volume with constant agitation speed (200 and 240 rpm) (**b**). Each data point represents the average of measurements performed simultaneously in three different 50-mL shake tubes ($n = 3$). The liquid phase was pure water. Experiments were carried out at 37 °C

$$a = \frac{S}{V}$$

a : specific interfacial area [m^{-1}], S : interfacial area [m^2]
V : liquid volume [m^3]
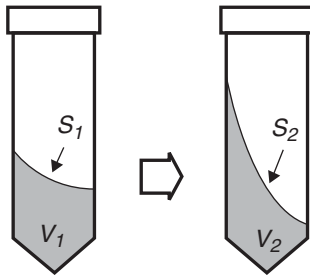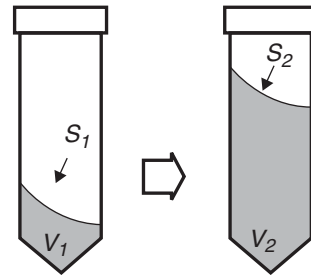
Fig. 5a,b Schematic diagram of the impact of variation in agitation speed (**a**) and working volume (**b**) on the specific liquid mass transfer coefficient $(k_L a)$ in 50-mL shake tubes

with the increased gas–liquid interface area *(S)* that resulted at higher shaking speeds. This is schematically shown in Fig. 5. The liquid mass transfer coefficient k_L was probably increased at higher agitation speeds as well. k_L depends on the diffusion of the gas in liquid, which might be favored at increased agitation speeds ([17]. Hermann et al. assessed the variations of the k_L in deep-well plates with liquid volumes of 200 mL [27].

k_L was found to be more or less constant in deep-wells with a round cross-section (0.2 m h^{-1}). A small increase was observed at agitation speeds above 600 rpm.

In bubble aeration systems, such as stirred-tank and airlift bioreactors, the specific interfacial area is given by the number and size of the bubbles that are generated. In surface aeration systems, the oxygen transfer occurs over the liquid surface only, resulting in poor oxygen transfer rates as is the case in spinner flasks. However, investigations made with shake flasks and now with 50-mL shake tubes show that generating a highly dynamic interface with an increased exchange surface (and surface renewal rate) is sufficient to achieve optimal oxygen transfer properties in small-scale systems.

Next, the working volume in 50-mL tubes was varied from 15 to 45 mL. A more than twofold $k_L a$ decrease at both 200 and 240 rpm was observed (Fig. 4b). However, even with a working volume of 45 mL (90% of the nominal tube volume), a relatively high $k_L a$ of approximately 8 h^{-1} resulted, more than sufficient to supply oxygen levels for a culture density equivalent to those in highly optimized stirred tanks. The decrease of the $k_L a$ was correlated with the reduction of the specific interface area a (Fig. 5). At a constant agitation speed, the interfacial surface (S) is expected to be approximately the same for different volumes. Therefore, when using larger working volumes a is less favorable than with smaller volumes. In such a case, a higher shaking speed could be used to maintain an adequate oxygen transfer rate.

The oxygen transfer characterization in 50-mL shake tubes revealed that a wide range of $k_L a$ values could be obtained when varying the shaking speed or the working volume. The upper limit of $k_L a$ values was close to 50 h^{-1} and might be higher when using lower working volumes such as 5 and 10 mL. Thus, 50-mL shake tubes were shown to deliver continuously sufficient oxygen to cells in suspension, ensuring a non-limiting rate of oxygen transfer. This explains the high cell densities (more than 1×10^7 cells mL^{-1}) frequently observed in these vessels.

3.2   Maintenance of Adequate OTR During Scale-Up of Shake Bioreactors

As mentioned above, surface aeration rather than sparging is preferred in shaken bioreactors. This normally results in a decreased specific oxygen transfer area as the working volume increases [28]. As a consequence, achieving a sufficiently high OTR for the optimal growth of cells is one of the key challenges in the scale-up of shaken bioreactors for the large-scale cultivation of mammalian cells.

Operational parameters that have a considerable impact on the shaken bioreactor's performance include the reactor size and shape, the orbital shaking speed, the shaking diameter, the filling volume, and the surface properties of the vessel material [5, 17]. Functioning as the "agitator" of stirred-tank reactors, the vessel wall of the shaken bioreactor, with its particular geometry (cylindrical or square-shaped) in addition to the size and shape of interior baffles, determines the mixing behavior and the oxygen transfer capacity at any given shaking speed and working volume.

Table 1 k_La values measured in shaken bioreactors with different geometries

Nominal volume [L]	Geometry	Working volume [L]	Shaking speed [rpm]	Aeration	k_La [h^{-1}][a]
Bench-scale					
0.05	Cylindrical	0.01–0.02	180–220	Passive	5–30
0.05	Cylindrical, helical track	0.01–0.02	180–220	Passive	70–100
1	Cylindrical	0.2–0.4	110	Passive	5–10
1	Square-shaped	0.4	110	Passive	15
1	Cylindrical, helical track	0.3	110–120	Passive	20–30
Pilot-scale					
10	Square-shaped	5	65	Active	15
20	Square-shaped	10	80–82	Active	5–8
30	Cylindrical	15	80–120	Active	2–8
30	Cylindrical, helical track	15	70–120	Active	5–70
Large-scale					
200	Cylindrical	100	65–75	Active	2–8
2,000	Cylindrical	1,000	45–48	Active	2–3
1,500	Cylindrical, helical track	1,000	40	Active	10

[a]The k_La values were determined by the method mentioned above. Typically the working volume was 30–60% of the nominal volume. Shaking speeds were empirically set at values that ensured homogeneous mixing without producing excessive turbulence and foaming

For cylindrical shaken vessels at moderate shaking speeds, a k_La value around 8 h^{-1} was obtained at working volumes up to 100 L (Table 1). By using square-shaped vessels of the same scale [5, 8], the oxygen transfer capacity was typically enhanced one- to two-fold (Table 1). At scales beyond 100 L, cylindrical vessels were found to be more suitable for cell cultivation because the fluid pattern in these vessels is more predictable and thus more scalable than in square-shaped vessels. At larger scales, the lower k_La values in the cylindrical vessels can be compensated by using oxygen-enriched air or pure oxygen once the cell density is sufficiently high.

Further improvement of oxygen transfer was achieved by introducing a helical track into a cylindrical vessel (Fig. 6). With a helical track on the inside wall (Fig. 6a), the liquid in the cylindrical vessels developed a rotating flow pattern due to orbital shaking with a maximal velocity proportional to the shaking frequency and the vessel diameter. This velocity provided the kinetic energy needed to "pump" liquid onto the helical track and towards the oxygen-rich headspace of the reactor, thus increasing the liquid-gas interface area to a significant extent. This typically resulted in a 5- to 10-fold improvement in the k_La (Table 1). Even in a 1,500-L helical track vessel with a working volume of 1,000 L, a surprisingly high k_La value of 10 h^{-1} was obtained at a relatively low shaking speed of 39 rpm and an axial displacement radius of 10 cm (Table 1). This result opens the possibility of supporting the high-density cultivation of mammalian cells at volumetric scales up to 1,000 L using only surface aeration with air.
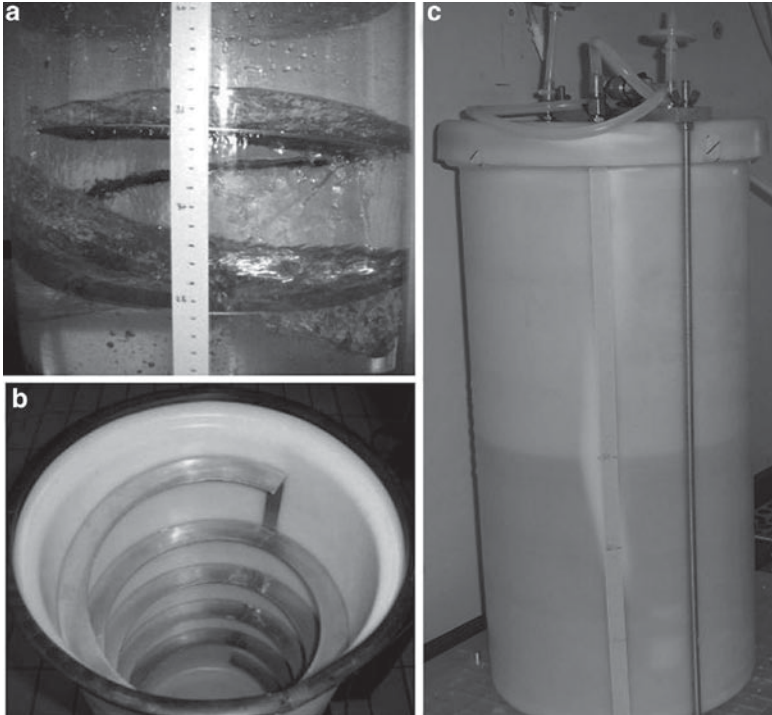
Fig. 6a–c Cylindrical helical track vessels designed to improve the oxygen transfer capacity of shaken bioreactors. **a** The liquid was "pumped" to the top end of a helical track in a 30-L cylindrical vessel at a shaking speed of 90 rpm. The vessel had a diameter of 30 cm and height of 45 cm. The removable stainless steel helical track had 3.5 turns with a width of 3.0 cm and a pitch of 9 cm. The fast-moving liquid on the track extended the oxygen transfer area and thus improved the $k_L a$ of the shaken vessel. **b,c** The diameter and height of the 55-L helical track bioreactor were 33 cm and 66 cm, respectively. The stainless steel track had five turns with a width of 3.6 cm and a pitch of 10 cm. The typical working volume of this 55-L bioreactor was 30–40 L with a shaking speed of 70–80 rpm. The system was aerated by active surface aeration with air at a constant flow rate of 1 L min^{-1} corresponding to 0.025 vvm (volume of air per volume of medium per minute)

4 Development of Scalable Shake Bioreactor Technologies

Flasks and bottles up to 2 L, disposable or not, are nowadays widely used to cultivate animal cells in suspension. Incubators with orbital shakers are used to maintain physiological conditions and homogeneous mixing. To ensure sufficient renewal of the air in the headspace while preventing contamination, caps fitted with sterile filters are used. However, investigations focused on volumetric scales beyond 10 L are scarce. Liu and Hong published pioneering work in this field [3]. They reported the design of shake bioreactor systems for animal cell cultures using a shaker and a cylindrical vessel of variable size with a height to diameter ratio of 3:2.

In fed-batch cultures, CHO cells were grown to 6×10^6 cells mL^{-1} in a 56-L shake vessel containing 18 L of cell suspension. The study stressed the potential utility of this simple cost-effective bioreactor system for the cultivation of mammalian cells. More recently, the system was successfully extended to the cultivation of insect and plant cells in suspension [29]. In this study, the mixing time in a 50-L bioreactor with a 35 L working volume varied between 100 and 10 s when the agitation speed increased from 80 to 120 rpm.

The possibility of using even larger disposable shake containers was suggested by Büchs and coworkers [30]. The power consumption of large rotary shaking vessels with agitation speeds of 100–300 rpm was investigated. In large shake systems (20 L nominal volume), the power consumption per unit volume was in the same range as that of small shaking flasks (nominal volume less than 2 L). However, no study addressing the question of oxygen supply in larger orbital shake vessels has been published. Modelling approaches aimed at predicting the oxygen transfer in shake flasks were recently proposed [17, 31, 32]. Though developed for small-scale systems, such models might be useful for the development of large-scale shake bioreactors.

An overview of recent research and development efforts made in our labs to scale-up shake technology for animal cell cultivation is provided here. To confirm the promising features of orbital shake technology at larger scales and to reach a balance between mass transfer requirements and shear sensitivity of cells, various prototype shake bioreactors were designed and tested.

For nominal volumes of up to 20 L, autoclavable disposable polycarbonate containers were used (Fig. 7a). For larger volumes, helical track vessels (Fig. 6b) and presterilized disposable bags and containers (Fig. 7b) were tested. Instead of passively exchanging air, like in 50-mL shake tubes, larger systems were actively
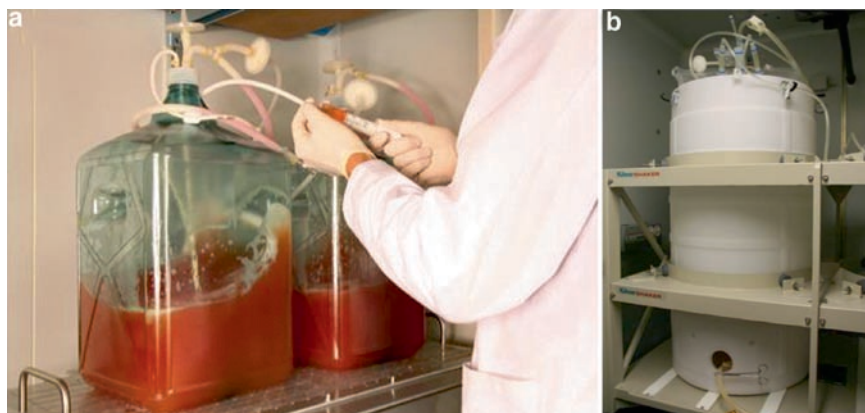
Fig. 7a,b Square-shaped disposable shake containers for volumes up to 20 L on a benchtop orbital shaker (maximal working volume of 10 L) (**a**) and disposable cell culture bags for volumes up to 200 L (maximal working volume of 100 L) (**b**). The bags were fitted into a cylindrical-shaped container that was orbitally agitated on a large-capacity shaker

aerated to eliminate the transfer resistance in the gas phase. A given airflow rate was provided to replace gas in the head space of the reactor. If needed, the airflow was enriched with pure oxygen to increase further the OTR. Despite a reduced specific area of the gas–liquid interface in larger systems, optimal conditions were identified which resulted in growth kinetics approaching those observed in 50-mL TubeSpin®-bioreactors.

4.1 The 10- and 20-L Square-Shaped Containers

Initially, CHO DG44 cells were cultivated in 10- and 20-L square-shaped containers agitated on orbital benchtop shakers (Fig. 8). Repeatedly, cell densities of 6×10^6 cells mL^{-1} were obtained at both scales in batch cultures. Typically, the viability was maintained above 90% during the exponential growth phase and declined rapidly after a short stationary phase. To ensure sufficient oxygen transfer, air was continuously added to the headspace at a flow rate of 1 L min^{-1}. The pH was adjusted by varying the CO_2 concentration in the inlet airflow. Normally, 5% CO_2 was added for 1 or 2 days. At higher cell densities, CO_2 was not added to the inlet airflow due to an increased cellular CO_2 release. The agitation speed was empirically set at values that ensured homogeneous mixing without producing excessive turbulence and foaming. Similar growth kinetics and viabilities were observed in square-shaped and cylindrical containers. To maintain the cells in suspension, higher agitation speeds were required in cylindrical containers of the same volume (95–100 rpm instead of 74–76 rpm at the 20-L scale). A recent study demonstrated
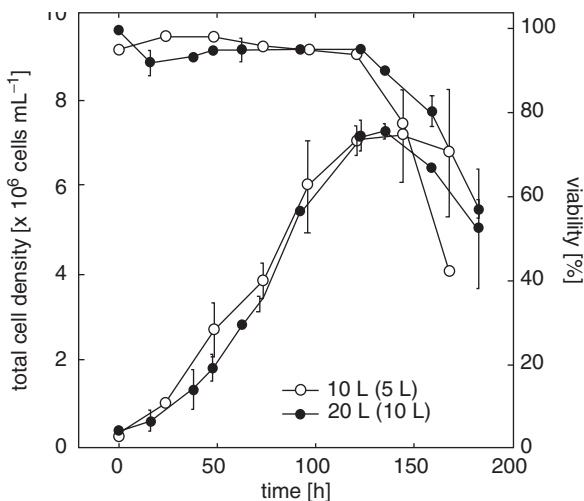
Fig. 8 CHO DG44 batch cultures in 10- and 20-L shake bioreactors operated with working volumes of 5 and 10 L, respectively, and agitation speeds of 69 and 76 rpm, respectively. The airflow rate was set at 1 L min^{-1} for both systems. Average cell densities and viabilities are reported ($n = 2$)

that at the same volumetric power consumption, the maximum local energy dissipation rate in baffled and in nonbaffled shake flasks was similar [33]. Consequently, cultures in cylindrical and square-shaped containers of the same volume might be exposed to comparable shear stress conditions when operated at different agitation speeds but at the same power consumption, resulting in similar growth kinetics.

Growth performance in a disposable shake bioreactor was compared with a stirred-tank bioreactor of the same volume (20-L stirred-tank bioreactor, Bioengineering AG, Wald, Switzerland). The experiment was performed with batch cultures of recombinant CHO cells (CHO-AMW) that express a human IgG monoclonal antibody. When cell densities reached 5×10^6 cells mL^{-1}, the temperature was reduced to 31 °C to extend the viability and enhance IgG expression. The initial cell density was approximately 3×10^5 cells mL^{-1} in both systems. As shown in Fig. 9, similar growth performances were observed in the two bioreactor systems. Though a longer lag-phase was seen for the culture in the shake bioreactor, the overall growth kinetics during the exponential phase were nearly identical in the two systems. Cells reached the target density for the temperature reduction about 10 h earlier in the stirred-tank bioreactor than in the shake bioreactor. This had a negligible consequence on the final IgG yield as concentrations above 100 mg L^{-1} were observed in both cases (data not shown). A fine-tuning of the shaking speed at the very beginning of the cell culture may reduce the length of the lag phase in the shake bioreactor.
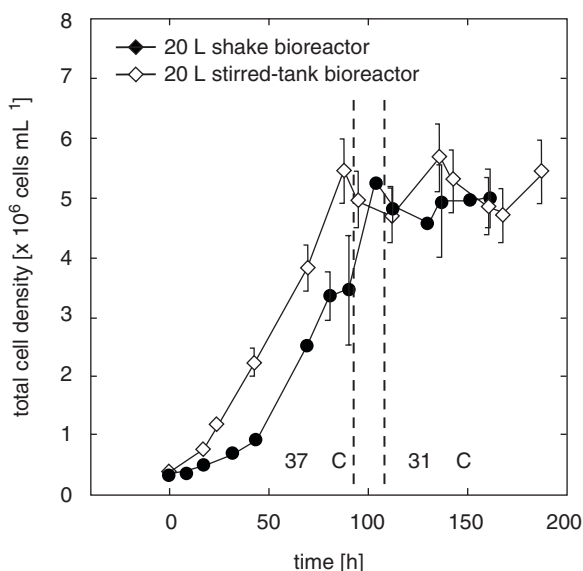
Fig. 9 CHO-AMW batch cultures in 20-L stirred-tank and shake bioreactors with working volumes of 10 L. The temperature was shifted from 37 to 31 °C when densities reached 5×10^6 cells mL^{-1}. The *dotted vertical lines* represent the temperature shift for the stirred-tank bioreactor (97 h) and the shake bioreactor (106 h), respectively. Viability in both bioreactors was above 90% until the temperature was shifted and declined afterwards. Average densities are reported ($n = 2$)

Actively aerated 10- and 20-L shake bioreactors were found to be ideal for rapid and efficient cell expansion. They require less setup time than stirred-tank bioreactors of the same volumes and the handling is simplified. Standard lab equipment was used to operate such systems, which is a distinct advantage over more conventional bioreactor systems. Though intended for single-use, polycarbonate containers can be cleaned, autoclaved, and reused several times, further reducing the operating costs. Also, multiple shake bioreactors can be operated simultaneously, which is often desirable when optimizing and scaling up a process.

4.2   The 55-L Cylindrical Helical Track Container

With the measured $k_L a$ values in the helical track vessels described above, a sufficient oxygen supply from active surface aeration with air was expected for the cultivation of mammalian cells at densities $>10^7$ cells mL^{-1} even at the 1,000-L scale (Table 1). To test the feasibility of applying helical track vessels to the cultivation of mammalian cells, suspension cultures of CHO DG44 and HEK 293 cells were grown in a cylindrical 55-L helical track vessel. The batch and fed-batch cultivation of CHO DG44 and HEK 293 cells in a cylindrical 55-L helical track bioreactor with a working volume about 40 L resulted in improved cell growth profiles compared to control cultures in 50-mL tubes and in a 28-L stirred-tank bioreactor. Growth performance of a fed-batch culture of CHO DG44 cells is shown as an example. While flushing the head-space of the reactor with about one reactor volume of air per hour (1 L min^{-1}, i.e., 0.025 vvm – volume of air per volume of medium per minute), the OTR was never rate-limiting as the DO level remained about 90% even at the maximum cell density of 9.3×10^6 cells mL^{-1} (Fig. 10). The viability remained above 95% for the entire cultivation period. Theoretically this reactor can therefore support cell densities much higher than 10^7 cells mL^{-1} with surface aeration using air only.

4.3   Scales Beyond 100 L

The promising data described above was a strong driver for the design and testing of shaken vessels at larger operating scales. Oxygen transfer tests were initially performed at the 200-L scale. The tests were also useful to assess the reliability of the shakers and to define the operable combinations of working volume and agitation speed. Experiments were performed with pure water to assess the time required to heat up the shake bioreactors to 37 °C. In stirred-tank bioreactors, the use of steam and heating jackets results in brief heat-up times. Similar heat exchange systems were not available for the shake bioreactors, described here. At the 200-L scale, a simple and lightweight heating system was tested. Silicon heat elements (Hotsil, Prang + Partner AG, Pfungen, Switzerland) were inserted between the
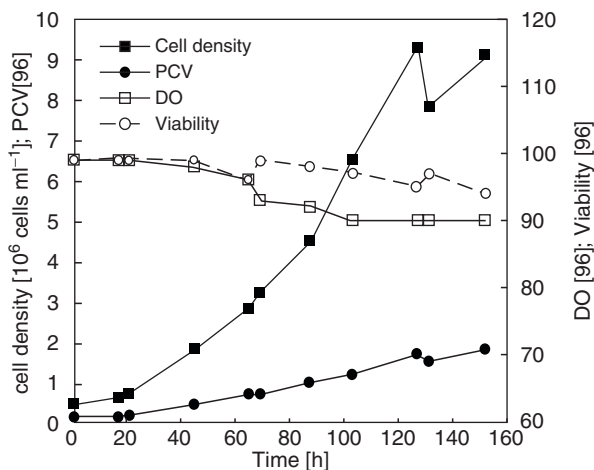
Fig. 10 Fed-batch cultivation of CHO DG44 cells in a 55-L cylindrical helical track bioreactor. The culture was inoculated with a working volume of 27 L and a cell density of 0.4×10^6 cells mL^{-1}. The shaking speed ranged from 70 at the time of inoculation to 82 rpm at 120 h post-inoculation. The surface aeration rate was 0.025 vvm. 3 L and 5 L of 2 × concentrated ProCHO 5 medium were added to the culture at 72 and 96 h post-inoculation, respectively. At 120 h post-inoculation, 7 L of ProCHO 5 medium were added. The final working volume was 42 L. DO, viable cell density, and viability were measured at the times indicated

container wall and the cell culture bag. For a liquid volume of 100 L and an agitation speed of 75 rpm, heating times of 8–10 h were observed. The use of heating elements with larger contact surfaces and with improved insulation is expected to shorten the time necessary for heating.

Cell cultivation in a 200-L container was evaluated using CHO-AMW cells, first with a working volume of 25 L and then with 100 L. As a control, a 150-L stirred-tank bioreactor was inoculated with 50 L and operated as a standard batch procedure. The shake bioreactors and the control bioreactor were started with initial densities of $3-5 \times 10^5$ cells mL^{-1} [11]. In all cultures the cells grew to densities up to 6×10^6 cells mL^{-1} by 125 h after inoculation. During the exponential growth phase, the viability was higher than 90% in all three cultures (Fig. 10). To ensure sufficient oxygen transfer when operated with 100 L, the airflow for the 200-L container was enriched with pure oxygen (20–50%) at the higher cell densities (3×10^6 cells mL^{-1}). The results from the comparison with the 150-L stirred-tank bioreactor were promising since similar growth trends and viabilities were observed in the 200-L container. As seen before at the 20-L scale, a somewhat longer lag phase in the shake bioreactor delayed the time when the maximal cell density was reached as compared to the stirred-tank bioreactor (Fig. 11). Importantly, none of the systems tested here were systematically optimized in a way that would normally be done for production purposes.

Today, large-scale mammalian cell culture is almost exclusively performed in stirred-tank bioreactors. However, we believe that suspension cell culture using
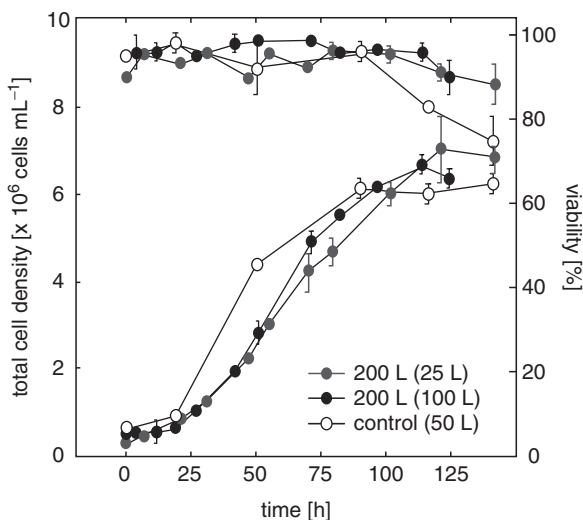
Fig. 11 CHO-AMW batch cultures in a 200-L shake bioreactor with 25 and 100 L working volumes. The shaking speed was set at 50 and 65 rpm, respectively. The airflow rate was 1–2 L min^{-1} in both cases. At the 100 L working volume, when the density was above 3×10^6 cells mL^{-1}, the airflow was enriched with 20–50% oxygen. Cell growth was compared with a batch culture in a 150-L stirred-tank bioreactor (*control*) with 50 L working volume. The DO for this culture was maintained above 20% using oxygen-enriched air. Average cell densities and viabilities are reported for the times indicated ($n = 2$)

orbital shaken bioreactors will become an attractive option at scales up to 1,000 L because of increased flexibility and reduced costs [3, 5, 11]. For further testing of this principle, we have designed and manufacturer a prototype 2000-L shaken bioreactor that can be fitted with disposable cultivation bags (Fig. 12). The k_La values and the successful cell growth described here for shaken reactors imply that these vessels meet all the essential requirements for a cell culture system: mixing without damaging shear-sensitive cells, sufficient gas transfer, and ease of scale-up and process control [34]. Although operating conditions in large-scale shaken bioreactors require further exploration, the data presented here demonstrate the potential of this simple bioreactor for applications to large-scale high-density mammalian cell cultivation.

5 Further Perspectives and Potential Commercial Applications

Although disposable, single-use technologies are now widespread in many process steps including filtration, sterile liquid handling, media and buffer preparation, the standard equipment for cell cultivation remains non-disposable. Stirred tank and airlift bioreactors were initially developed for microbial production systems to achieve high gas transfer using direct gas dispersion into the liquid phase. For mammalian

Fig. 12 A 2,000-L disposable shaken bioreactor system with a working volume up to 1,000 L. Shaking diameter: 10 cm, shaking speed: 40–45 rpm

cells, however, the design and the positions of impellers and spargers were modified to reduce the hydrodynamic shear conditions, resulting in less efficient gas transfer properties [34]. Other drawbacks include the substantial capital investment required to equip a manufacturing plant. With stainless steel bioreactors, each new production batch requires a time-consuming testing procedure following sterilization in place (SIP). Similarly, equipment setup and cleaning during campaign changeovers in multi-product facilities require considerable time. Such downtimes result in poor overall manufacturing productivities. In this respect, the lack of flexibility with stainless steel bioreactors represents a risk in terms of future process volume estimations.

To overcome most of the disadvantages associated with stainless steel bioreactors, efforts are being made to develop single-use cultivation systems. This trend was initiated at small-scale with the development of disposable shake flasks and tubes for cell culture together with appropriate incubator shakers. These non-instrumented systems were found to be reliable for cell line development applications [35]. For larger processing volumes, disposable bioreactors based on single-use bags were developed. In 1998 Wave Biotech was the first company to commercialize a completely disposable cell cultivation system. The system is now widely accepted for many applications at scales up to 500 L, particularly as a cell expansion system to inoculate stirred-tank bioreactors. This success story created new market opportunities for

innovative cell culture systems. In fact, a number of other designs quickly entered the market, such as single-use stirred-tank and air-lift bioreactors, based on disposable bag technology. Although disposable bags are used in these novel bioreactor systems, they employ different mixing techniques and have distinct flow patterns, all aimed at obtaining an adequate gas transfer capacity.

Orbitally shaken vessels, especially shake flasks, have been the most widely used bioreactor system for over half a century, mainly for microbial cultures. Due to the high oxygen consumption rate of microbial hosts, the working volume of these shaken bioreactors is limited to 10–20% of the nominal volume. Compared to bacteria, cultivated mammalian cells have a relatively low demand for oxygen. Importantly, concerns about the shear sensitivity of mammalian cells have been somewhat lessened by cell engineering and the continuous optimization of the medium during the last few decades, opening up the possibility of culturing mammalian cells in large scale shaken bioreactors. In our lab, shaken bioreactors have been used successfully to culture CHO cells to scales up to 1000 L, but we feel this may not be the highest scale possible.

Despite the perceived importance and potential of disposable bioreactors, several major issues are still not solved. For example, a better understanding of the engineering principles of these vessels is needed. By comparison, the fluid mixing, mass transfer capacity, power input, and shear stress in stirred-tank bioreactors has been extensively studied for decades. This knowledge base benefits process development and scale-up. Their well-defined and well-controlled environment allows efficient process monitoring, which is very important for biopharmaceutical productions. To attempt to close this knowledge gap between the two systems, investigations on the special mixing pattern obtained by orbital shaking are now underway with the help of CFD. The free-surface shapes and the velocity fields obtained in the simulations compared well with experimental data (Fig. 13). Understanding these parameters is necessary for the scale-up of shaken bioreactors and further process optimization. Also, a better understanding of engineering issues associated with shaken bioreactors is expected to provide a confidence boost with regard to the acceptance of this new type of bioreactor.

Another question concerns the largest possible operational scale for orbital shaking. Technical problems like achieving sufficient gas-liquid transfer capacity at an adequate shaking speed is the main concern for the scale-up of orbitally shaken bioreactors. An increase in shaking speed clearly results in an improved oxygen transfer capacity. In a 2000-L cylindrical disposable bag with a filling volume of 1000 L, a $k_L a$ value of nearly 3 h^{-1} was observed at 47 rpm (Table 1), nearly twice as high as the $k_L a$ value obtained at 45 rpm (1.5 h^{-1}) and three times higher than that at 43 rpm (1 h^{-1}). A $k_L a$ value of nearly 3 h^{-1} could theoretically support a batch culture using surface aeration with air. However, a balance between mass transfer and the shear sensitivity of the cells has to be considered. In mammalian cell culture, hydrodynamic stress may limit the maximal cell density [36]. It has previously been reported that at the same volumetric power consumption, the maximum energy dissipation rate in shake flasks is about ten times lower than in stirred-tank reactors [33]. Together with the preliminary data of the 1,000 L CHO culture, this observation
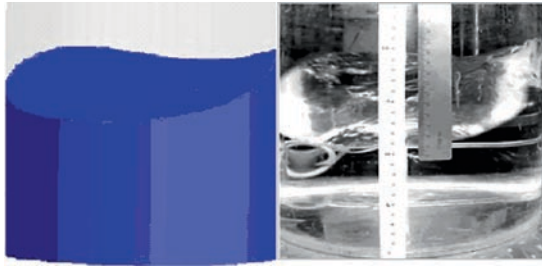
Fig. 13 Free surface in a 30-L cylinder with a working volume of 12 L and an orbital shaking speed of 80 rpm. The surface simulated by CFD (*left*) and the actual free surface (*right*) were nearly identical. The analysis of the velocity field and shear stress derived from the same model are underway

demonstrates the real possibility of expanding the orbital shaking technology to scales beyond 1,000 L. However, additional studies with CFD simulations and with cell cultures are necessary to confirm this preliminary observation.

The $k_L a$ and cell cultivation results of helical track vessels open additional opportunities to develop and improve processes further with disposable shaken bioreactors. By this innovative mixing principle, a much higher – typically tenfold – oxygen transfer capacity was achieved, meaning a much lower shaking speed could be used to obtain a sufficient OTR compared to the cylindrical vessel without a helical track. The powerful oxygen transfer capacity of helical track vessels will undoubtedly allow them to be used for bacterial fermentations as well. At 120 rpm, a $k_L a$ value higher than 70 h^{-1} was obtained in a 30-L shaken helical track vessel with a working volume of 50% of the nominal volume. Preliminary experiments with *E. coli* cultures have shown that a 55-L helical track vessel with a working volume of 30 L shaken at 80–90 rpm had the same performance as a 5-L baffled shake flask with 2 L working volume shaken at 120 rpm.

Taking advantages of breakthroughs in disposable technologies, especially bags and sensors for pH and DO monitoring, appropriate and reliable shaken bioreactor systems that would ideally support high cell density cultures at scales beyond 1,000 L are expected to be developed in the near future. This step is in line with the recent trend in the biopharmaceutical industry towards disposable, single-use bioreactor technology for cost-effective and flexible recombinant protein production.

References

1. Wurm FM (2004) Nat Biotechnol 22:1393
2. Büchs J (2001) Biochem Eng J 7:91
3. Liu C, Hong L (2001) Biochem Eng J 7:121
4. De Jesus MJ, Girard P, Bourgeois M, Baumgartner G, Jacko B, Amstutz H, Wurm FM (2004) Biochem Eng J 17:217
5. Muller N, Girard P, Hacker DL, Jordan M, Wurm FM (2005) Biotechnol Bioeng 89:400

6. Micheletti M, Barrett T, Doig SD, Bagany F, Levy MS, Woodley JM, Lye GJ (2006) Chem Eng Sci 61:2939
7. Muller N, Derouazi M, Van Tilborgh F, Wulhfard S, Hacker DL, Jordan M, Wurm FM (2007) Biotechnol Lett 29:703
8. Stettler M, Zhang XW, Hacker DL, DeJesus MD, Wurm FM (2007) Biotech Prog 23:1340
9. D'Aquino R (2006) Chem Eng Prog 102:8
10. Girard P, Jordan M, Tsao M, Wurm FM (2001) Biochem Eng J 7:117
11. Stettler M, De Jesus M, Ouertatani H, Engelhardt E-M, Muller N, Chenuet S, Bertschinger M, Baldi L, Hacker D, Jordan M, Wurm FM (2007) 1,000 non-instrumented bioreactors in a week. In: Smith R (ed.) Cell technology for cell products. Springer, Dordrecht, The Netherlands
12. Derouazi M, Girard P, Van Tilborgh F, Iglesias K, Muller N, Bertschinger M, Wurm FM (2004) Biotechnol Bioeng 87:537
13. Stettler M, Jaccard N, Hacker D, DeJesus M, Wurm FM, Jordan M (2006) Biotechnol Bioeng 95:1228–1233
14. Pham PL, Perret S, Doan HC, Cass B, St-Laurent G, Kamen A, Durocher Y (2003) Biotechnol Bioeng 84:332–342.
15. Pham PL, Perret S, Cass B, Carpentier E, St-Laurent G, Bisson L, Kamen A, Durocher Y (2005) Biotechnol Bioeng 90:332
16. Krahe M (2002) Biochemical Engineering. In: Ullmann's Encyclopedia of Industrial Chemistry. 6th Edition. pp. 171-240. Wiley-VCH Verlag GmbH & Co., Weinheim, Germany
17. Maier U, Büchs J (2001) Biochem Eng J 7:99
18. Zhang H, Williams-Dalson W, Keshavarz-Moore E, Shamlou PA (2005) Biotechnol Appl Biochem 41:1–8
19. Deshpande RR, Heinzle E (2004) Biotechnol Lett 26:763
20. Deshpande RR, Koch-Kirsch Y, Maas R, John GT, Krause C, Heinzle E 2005) Assay Drug Dev Technol 3:299
21. Kensy F, Zimmermann HF, Knabben I, Anderlei T, Trauthwein H, Dingerdissen U, Büchs J (2005) Biotechnol Bioeng 89:698
22. Gupta A, Rao G (2003) Biotechnol Bioeng 84:351
23. Wittmann C, Kim HM, John G, Heinzle E (2003) Biotechnol Lett 25:377
24. Amoabediny G, Büchs J (2007) Biotechnol Appl Biochem 46:57
25. John GT, Klimant I, Wittmann C, Heinzle E (2003) Biotechnol Bioeng 81:829
26. Suijdam JCV, Kossen NWF, Joha AC (1978) Biotechnol Bioeng 20:1695
27. Hermann R, Lehmann M, Büchs J (2003) Biotechnol Bioeng 81:178
28. Marks DM (2003) Cytotechnology 42:21
29. Raval K, Liu C, Büchs J (2006) BioProcess Int 4:46
30. Kato Y, Peter CP, Akgün A, Büchs J (2004) Biochem Eng J 21:83
31. Nikakhtari H, Hill G (2005) Can J Chem Eng 83:493
32. Büchs J, Maier U, Lotter S, Peter CP (2007) Biochem Eng J 34:200
33. Peter PC, Suzuki Y, Buchs J (2006) Biotech Bioeng 93:1164
34. Varley J, Birch J (1999). Cytotechnology 29:177
35. Morrow J (2006) GEN 26:52
36. Senger RS, Karim MN (2003) Biotechnol Prog 19:1199

Bag Bioreactor Based on Wave-Induced Motion: Characteristics and Applications

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Abstract Today wave-mixed bag bioreactors are common devices in modern bio-technological processes where simple, safe and flexible production has top priority. Numerous studies that have been published on ex vivo generation of cells, viruses and therapeutic agents during the last 10 years have confirmed their suitability and even superiority to stirred bioreactors made from glass or stainless steel for animal as well as plant cell cultivations. In these studies the wave-mixed bag bioreactors enabled middle to high cell density and adequate productivity in laboratory and pilot scale. This mainly results from low-shear conditions and highly efficient oxygen transfer for cell cultures, as demonstrated for the widely used BioWave®.

Starting with an overview of wave-mixed bag bioreactors and their common operation strategies, this chapter delineates engineering aspects of BioWave®, which like Wave Reactor™ and BIOSTAT®CultiBag RM originates from the prototype of a wave-mixed bag bioreactor introduced in 1998. Subsequently, the second part of the chapter focuses on reported BioWave® applications. Conditions and results from cultivations with animal cells, plant cells, microbial cells and nematodes are presented and discussed.

Keywords Animal cells, Biological insecticides, Biotransformation, Immuno-modulator, Plant cells, Seed inoculum, Wave-mixed bag bioreactor.

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Abbreviations

A_O	surface area of the fluid
A_q	hydraulic cross-section of culture bag
ADV	adeno-associated virus
BEV	baculovirus expression vector
BHK	baby hamster kidney
B	width of culture bag
BY-2	cultivar Bright Yellow-2 from the tobacco plant
C_1	correction factor considering influence of bag type, rocking angle, rocking rate and culture volume on volumetric flow rate
C_2	correction factor depending on bag type and describing the correlation of BioWaWave®'s Re_{mod} and stirred bioreactor's Re_{mod}
CAD	computer aided design
CD	chemically defined
CFD	computational fluid dynamics
CHO cells	Chinese hamster ovary cells
CO_2	carbon dioxide
D	dilution rate (ratio of volumetric flow rate to culture volume)
DO	dissolved oxygen
dw	dry weight
E-FL cells	embryogenic feline lung fibroblast cells
E. coli	*Escherichia coli*
E. neophadidis	*Erynia neophadidis*

fw	fresh weight
GMP	Good Manufacturing Practice
H	height of culture bag (inflated)
HEK cells	human embryogenic kidney cells
H. megidis	*Heterorhabditis megidis*
H. muticus	*Hyoscyamus muticus*
H. procumbens	*Harpagophytum procumbens*
h	liquid level of culture bag
i	culture bag geometry, given by the ratio of L to B
KCl	potassium chloride
k	rocking rate
$k_L a$	volumetric oxygen transfer coefficient
L	length of culture bag
l	characteristic length of culture bag
M	momentum
MDCK cells	Madin–Darby canine kidney cells
M. domestica	*Malus domestica*
MOI	multiplicity of infection or optimal ratio of virus particles per cell
MEV	mink enteritis virus
mab	monoclonal antibody
NaCl	sodium chloride
N. tabacum	*Nicotiana tabacum*
P. ginseng	*Panax ginseng*
P/V	power input per volume (specific power input)
Re	Reynolds number
Re_{mod}	modified Reynolds number
r	recombinant
rpm	revolution per minute
Sf	*Spodoptera frugiperda*
S. cerevisiae	*Saccharomyces cerevisiae*
SEAP	secreted alkaline phosphatase
S. feltiae	*Steinernema feltiae*
SH medium	Schenk and Hildebrandt medium
S.U.B.	Single Use Bioreactor
T 408	tobacco strain with uracil transporter-like protein
T. baccata	*Taxus baccata*
$TCID_{50}$	tissue culture infectious dose
TOI	optimal density of cells at infection
tPA	tissue plasminogen activator
U	true length of culture bag
V	culture volume
$\dot{V}$	volumetric flow rate
Vero cells	kidney epithelial cells from African green monkey
VOF method	volume of fluid method
V. vinifera	*Vitis vinifera*

vvm	volume per volume per minute
W	work
WUB	Wave and Undertow Bioreactor
w	fluid velocity
φ	rocking angle
τ	residence time distribution
ν	kinematic viscosity
θ_{95}	mixing time (time required to achieve 95% homogeneity)
μ_{max}	maximum specific growth rate
2D	two-dimensional
3D	three-dimensional

1 Introduction

In wave-mixed bag bioreactors, a one or two dimensional movement of the bioreactor's platform induces a wave in the sterile plastic bag containing culture medium and cells. In this way, mixing is facilitated while the surface of the culture medium is continuously renewed, and bubble-free surface aeration is accomplished. In order to guarantee optimum mass and energy transfer within the mechanically-driven inflated culture bag, essential parameters such as rocking rate, rocking angle, vibration frequency, temperature and aeration rate are adjustable. In spite of the identical working principle of the various wave-mixed bioreactors presented in Table 1, there are major differences concerning the platform movement and culture bag design (bag material, bag scale, bag dimension, type of employed sensor probes and filters).

BioWave®, Wave Bioreactor™ and BIOSTAT®CultiBagRM (Fig. 1) are based on the first wave-mixed laboratory bag bioreactor originally manufactured for animal cell cultures in the late 1990s. They are characterised by wide as well as common usage and are similar in design. While these three scalable systems facilitate DO (dissolved oxygen) and pH measurement and control by having optical sensors in the bag, the also one-dimensional rocking AppliFlex can be operated either with disposable or standard sensors. However, in the Tsunami® Bioreactor, where linear scale-up is realised by increasing the number of rocking platforms and culture bags, on-line control of DO and pH of the culture medium in the bags is not possible [31].

In the case of the CELL-tainer®, a combination of rocking and horizontal displacement is performed. This two-dimensional movement ensures a more efficient oxygen transfer (see Sect. 3.1) and better carbon dioxide exchange than in the wave-mixed bioreactors previously mentioned. For this reason, the CELL-tainer® is also suitable for microbial high-density cultures [33–35].

In Nestlé's Wave and Undertow Bioreactor, WUB, (see Chap. 5), the wave providing mixing and aeration is generated by raising the platform. WUB's design supports the growth and product expression (isoflavones and antibodies) of soya and tobacco suspension cells [36, 37]. Finally, the one-dimensional vibrating

Table 1 Overview of wave-mixed bag bioreactors

Platform movement	Wave-mixed bag bioreactor type	Culture volume (L)	References	Developed or manufactured by
Rocking, one-dimensional	BioWave®	0.05–300	[1–14]	www.wavebiotech.net
	Wave Bioreactor™	0.1–500	[15–29]	www.wavebiotech.com
	BIOSTAT® CultiBagRM	1–100	[30]	www.sartorius-stedim. com
	AppliFlex	1–25	[31]	www.applikon-bio.com
	Tsunami®Bioreactor	12–480	[32]	www.megainternational. com.hk
Rocking, two-dimensional	CELL-tainer®	0.5–15	[33–35]	www.cellutionbiotech. com
Raising, one- dimensional	Wave and Undertow Bioreactor (WUB)	10–100	[36, 37]	www.nestle.com
Vibrating, one-dimensional	No name	1.5–4	[38]	www.jacem.kilani@ utc.fr

Fig. 1 BIOSTAT®CultiBag RM, optical package (photograph provided by Sartorius Stedim)

motion concept (vibration frequency between 15 and 35 Hz) was used to develop a novel wave-mixed laboratory bioreactor [38]. First results (mixing times ranging from 30 to 80 s, $k_L a$ values between 7 and 80 h^{-1}) of its engineering characterisation indicate that successful growth of plant cells, animal cells and micro-aerophil strains is possible.

2   Common Operation Strategies Used in Wave-Mixed Bioreactors

The wave-mixed bag bioreactors described in the preceding section have been explored in batch, fed-batch, repeated fed-batch, continuous and continuous perfusion mode. In batch mode, which has the advantages of simplicity, reliability and flexibility, the culture volume of the bag is constant during the whole cultivation process, which means that no medium or cells are added or removed. Known limitations are overflow metabolism, possible substrate and product inhibitions, and lower space-time yields than in fed-batch and continuous cultivations.

In fed-batch mode, the most common operational procedure for wave-mixed bag bioreactors, culture medium or a concentrated feeding solution is added to the bag (Fig. 2a) periodically or continuously until the maximum culture volume (50% of total bag volume) is reached [4, 8, 30, 33–35]. During the cultivation time, an increase in culture volume in the bag is typical. The aim of fed-batch cultivation is to continue cell growth while nutrient concentrations and growth rate are kept at a predefined level, and accumulation of inhibitory by-products (e.g. lactate, ammonia) is reduced or even prevented. This feeding procedure is preferred for substrate-inhibiting processes and high cell-density cultivations.

In addition, a repeated fed-batch mode can also be realised. In contrast to the fed-batch operation described above, the culture medium is partially or completely
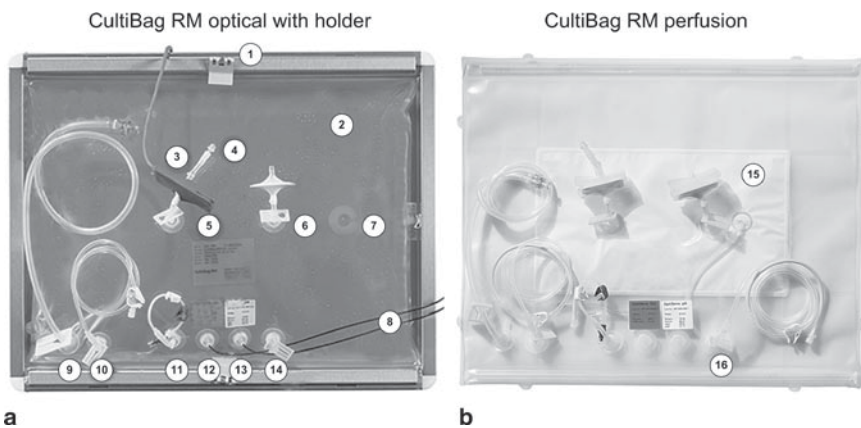
Fig. 2 Configurations of CultiBag RM: (**a**) CultiBag RM optical with holder: 1-holder; 2-culture medium; 3-filter heater, to prevent blocking of filter by condensation; 4-check valve; 5-gas filter outlet; 6-gas filter inlet; 7-temperature sensor on holder bottom; 8-optical fibres, signal transmission of sensors; 9-fill/harvest tubing, dip tube; 10-spare port, luer connector; 11-sample port, luer connector, samples are taken by resealable needle injection; 12-DO sensor, inbuilt disposable optical chemical sensor for DO; 13-pH sensor, inbuilt disposable optical chemical sensor for pH; 14-spare port, luer connector; (**b**) CultiBag RM perfusion: 15-internal fixed perfusion membrane; 16-perfusion outlet (photographs provided by Sartorius Stedim)

exchanged. Two-stage processes, where growth and production become independent by use of specific culture media (growth medium and production medium) differing in composition for the corresponding process phase, represent such repeated fed-batch cultivation procedures with complete medium exchange. At the end of the logarithmic growth phase, cell settlement (due to gravity) on the bottom of the culture bag takes place and fresh medium is added. The wave-mixed bag bioreactor is then left to run as a batch until the process is stopped [3, 7, 39]. To generate back-up cultures in seed inoculum production, a repeated fed-batch mode with partial medium exchange (and without cell retention) or an ordinary continuous mode is suitable.

If culture medium is continuously fed into the bag and the same amount of culture broth (medium and cells) is continuously removed (ordinary continuous mode), the liquid level in the bag is kept constant. The steady-state level of the cell population can be achieved as long as the chemostat principle (dilution rate $D \leq$ maximum specific growth rate μ_{max}) is guaranteed. Theoretically, continuous exponential cell growth can be maintained as long as desired, provided there is an optimum nutrient supply.

However, when $D > \mu_{max}$, the cells have to be retained in the bag and recirculated (biomass recirculation) in order to prevent cell wash-out. In combination with a weight-based or volume flow rate-perfusion controller, which balances culture medium renewal and harvest rate, specially designed bags with floating [21] or bottom-side fixed membrane [40] (see Fig. 2b) ensure successful internal perfusion up to 100-L culture volume. For culture volumes exceeding 100 L, external cell separation devices (cross-flow filtration units using hollow fibre modules, spin filters, gravity settlers, centrifuges, hydrocyclones – see also [41]) have to be used. In spite of the fact that the highest cell amounts, product titres and space-time yields can usually be achieved and inhibitory by-products in the spent culture medium can be removed efficiently, continuous perfusion cultivations are seldom realised. Perfusion cultures are only important when products are unstable or toxic to the cells, or substantial amounts of product (for example, for clinical studies) have to be generated within a short time. In the first instance, the unpopularity of perfusion mode can be explained by the higher levels of instrumentation and automation of the bioreactor facility as well as the risk of possible failure, which increases for operations over several weeks or even months. The validation procedure is therefore more complicated for processes subject to GMP demands.

3 Engineering Aspects: Biowave® as a Case Study

In addition to engineered cell lines as well as optimised culture medium and cultivation mode, advantages of BioWave® include its specific design and resultant gas transport efficiency, fluid flow, mixing characteristics, heat transfer and hydrodynamic shear pattern, all of which are recognised as key components in improving product quantity and quality. Whereas numerous studies (e.g. [42–51]) have been published for standard stirred bioreactors made from glass or stainless steel, only a limited

number [4, 33, 36, 38, 52, 53] are concerned with the engineering aspects of disposable bag bioreactors. This is quite problematic in the case of wave-mixed bag bioreactors, where insufficient engineering parameters make their comparison with well-known and well-accepted standard stirred bioreactors impossible. Only our studies focusing on BioWave®, which will subsequently be summarised, can be regarded as a first step in this direction.

3.1 Aeration and Oxygen Transfer Efficiency

As mentioned in the introduction, surface aeration supplying cells with oxygen and CO_2 is effected in BioWave®, where hydrodynamic stress for cells from bursting air bubbles is eliminated. It is common knowledge that aeration efficiency, evaluated by volumetric oxygen transfer coefficients ($k_L a$ values), depends mainly on the type of aeration (bubble aeration, surface aeration, membrane aeration, external aeration), culture volume, aeration rate and fluid flow of the cultivation system. Although bubble aeration can generate higher volumetric oxygen transfer coefficients, there is always the risk of damage to shear-sensitive cells from bursting air bubbles, which increases with greater bubble size. In the case of microsparging, stable foam layers can be formed which are resistant to antifoam agents and complicate downstream processing.

Volumetric oxygen transfer coefficients measured in BioWave® by using the dynamic gassing-out method (in water or cell culture medium at temperatures between 20 and 37°C) were comparable to or higher than those published for spinner flasks and the majority of cell-culture bioreactors operating with surface or bubble-free membrane aeration (see Table 2). These are in the range of typical $k_L a$ values in Newtonian-like animal cell culture media, which have been specified by numerous authors in Nienow [49] and lie between 1 and 15 h^{-1}. Our experimental set-up for dissolved oxygen measurement consisted of a sterilisable polarograph probe (Mettler Toledo) and a computer control and acquisition unit. Together with the calculation of $k_L a$ values, it is described in detail in [40] and [61].

Worthy of note is the superior oxygen mass transfer capacity (up to 700 h^{-1} [33]) determined for the wave-mixed CELL-tainer® with its additional horizontal displacement. We would like to point out that the incompleteness of the bioreactor's characteristic engineering parameters in numerous papers, and here especially of tip speed and power input, makes it difficult to compare the reference values of volumetric oxygen transfer coefficients summarised in Table 2. For example, Wave Bioreactor™'s volumetric oxygen transfer coefficients reported by Knevelman et al. [53] were a multiple higher than those determined in our experiments [40] for BioWave®. However, the direct relation of oxygen transfer efficiency to rocking rate, rocking angle and aeration rate was confirmed by both groups. For BioWave® and Wave Bioreactor™, it was demonstrated that high volumetric oxygen transfer coefficients can be ensured by an increase in rocking rate, rocking angle and aeration rate. At constant parameters, a decreased culture volume in the bag, resulting

Table 2 Volumetric oxygen transfer coefficients of different cultivation systems for cell cultures

Cultivation system	Culture volume (L)	Aeration type/-rate	Agitation	k_La (h^{-1})	References
Standard- and disposable stirred cultivation systems					
Spinner flask	1	Surface aeration	No stirring rate found	1	[19]
SuperSpinner (glass)	0.7	Surface aeration	35 rpm	0.4	[54]
		Membrane aeration, 0.14 vvm		1.9–3.5	
Bioreactor with eccentric tumbling stirrer	8	Membrane aeration, 0.2 vvm	100 rpm	4	[55]
Traditional stirred bioreactor	1.5	Membrane aeration, 0.1 vvm	175 rpm	5.2	[56]
	5	Bubble aeration, 0.1 vvm	100 rpm	10	[57]
		Microsparging, 0.1 vvm	100 rpm	50	[57]
	10	Bubble aeration, 0.25 vvm	200 rpm	15	[36]
Disposable stirred bioreactor, S.U.B.	50	Microsparging, 0.1 vvm	200 rpm	8.34	[58, 59]
	250	Microsparging, 0.5–2 L min^{-1}	100 rpm	7–15	[52]
Disposable wave-mixed cultivation systems					
AppliFlex	10	Surface aeration 0.25 L min^{-1}	4°, 20 rpm	4.4	[60]
		Surface aeration 0.5 L min^{-1}	6°, 20 rpm	8.4	
BioWave®	1	Surface aeration, 0.25 vvm	6°, 30 rpm	4	[40]
			10°, 30 rpm	10	
	10	Surface aeration, 0.25 vvm	5°, 30 rpm	9.3	
	100	Surface aeration, 0.04 vvm	10°, 24 rpm	5.6	
Wave Bioreactor™	10	Surface aeration, no aeration rate found	5°, 35 rpm	20	[53]
CELL-tainer®	10	Surface aeration, n.i.	n.i.	≥700	[33]
Vibrating wave-mixed bioreactor (no name)	1.5	Surface aeration, 0.1 vvm, 10 mbar of overpressure	Vibration frequency of 15 Hz	8	[38]
WUB	30	Surface aeration, 0.18 vvm	15°	9	[36]

n.i: no information

in increased specific surface area and power input, increases $k_L a$ values. Even small changes in the rocking rate and/or rocking angle can increase the $k_L a$ more significantly than raising the aeration rate.

In our experience, volumetric oxygen transfer coefficients exceeding 11 h^{-1} are solely achievable by aeration rates over 0.5 vvm or aeration with pure oxygen in BioWave® [4, 62]. However, to enhance the existing oxygen transfer limitation for aerobically growing microbial cultures in the bag, a modification of the exhaust air filter (filter area and arrangement) and the insertion of an aeration membrane or disposable spargers and baffles, as proposed by Mikola et al. [15], become necessary.

3.2 Power Input

The mechanically driven platform of BioWave® induces a wave whose development and propagation are the main key factors for mass and energy transfer efficiency in the culture bag and thus for its mixing and aeration characteristics. The required power input is manually adjustable via rocking angle, rocking rate, culture bag dimension and culture volume. In order to study and predict BioWave®'s specific power input (which enables hydrodynamic stress on cells in the wave-mixed bag to be assessed), six different modelling approaches were compared. As depicted in Fig. 3 four static models were generated: an inertia model, a momentum transport model, a model using transformation into thermal energy, and a model using electric power for the 2-L culture bag [4, 40]. Presupposing real flow behaviour in the 2-L culture bag, static model 3 is the most exact. In the same way as static models 1 and 2, it was developed by calculating the momentums that arose from analytical as well
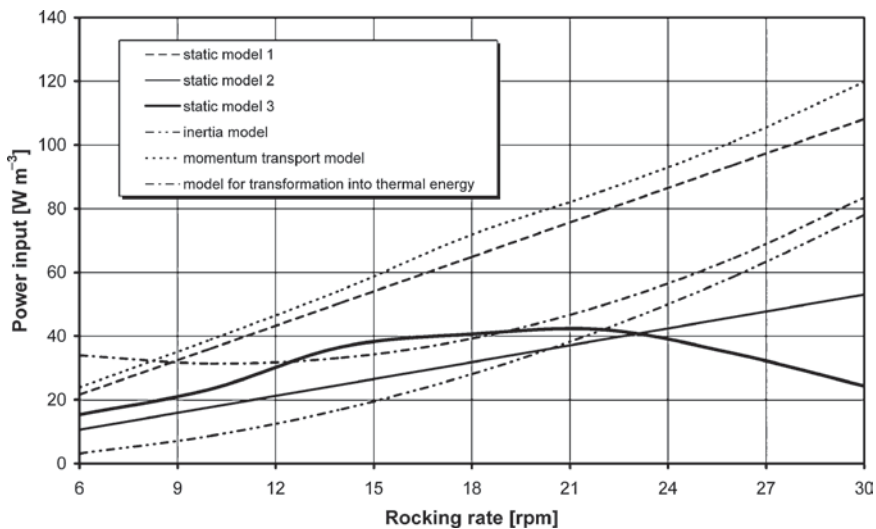
Fig. 3 Generated power input models for BioWave® 2-L culture bag (filling level 50%, rocking angle 10°)

a

b
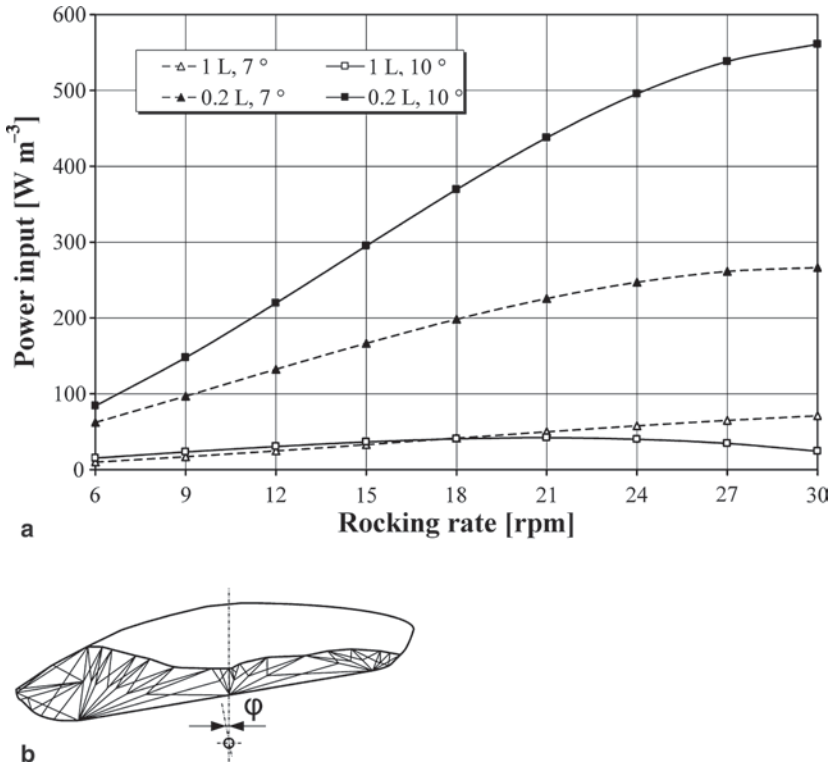
Fig. 4 Specific power input: (**a**) predicted on the basis of static model 3 for BioWave®'s 2-L culture bag; (**b**) determination of the point of gravity of the culture bag and the fluid surface

as graphical determination (see also Fig. 4b) of the point of gravity of the culture bag and the surface area of the fluid. On the basis of dynamic fundamental law (where a system only moves with constant velocity if the sum of all momentums acting on the system is in equilibrium), for all static models it is assumed that the fluid in the culture bag displays static behaviour. Observing a snap-shot of the cross-section of a filled and moving culture bag at different rocking angles, the fluid is distributed according to the rocking angle (φ), the culture volume (V) and the bag geometry (i) on both sides of the rotation point.

By determining the culture bag's point of gravity and the surface area of the fluid, the resulting momentums and also the necessary work can be calculated. The power input of BioWave® is analogous to the work required for one bidirectional rocking movement between the angles $-\varphi_{max}$ and $+\varphi_{max}$ and is given by (1).

$$W = \int_{-\varphi_{max}}^{+\varphi_{max}} M * \mathrm{d}\varphi \tag{1}$$

Equation (2), can be used to predict the specific power input as follows:

$$P\!\!\Big/\!\!_V = \frac{W * k}{V * 60} \tag{2}$$

As previously mentioned, superior static model 3 provided the basis for all our calculations of specific power input. Film sequences (30 per second) were taken to calculate the momentums. These film sequences, which were analysed using CAD (computer-aided design) software, showed the actual distribution of fluid during movement. In order to observe fluid movement, a 2-L culture bag was sliced along its longitudinal axis. A transparent plastic disc (complying in form and dimension to the cross-section of an aerated culture bag) was introduced into the half culture bag, which had been opened, and was subsequently glued to it. In addition, a scale printed on a foil was fixed on the plastic disc. This scale showed the angle and markers for a perpendicular bisector of the side, and fluid level at a rocking angle of 0° [40]. The specific power input values of BioWave® found in this way range from 8 to 561 W m^{-3} as shown in Fig. 4a.

Moreover, from Fig. 4a it becomes clear for the observer that minimum filling level, maximum rocking angle and rocking rate cause the maximum possible specific power input in 2-L culture bag, which is one decimal power higher than operation with maximum culture volume. It is obvious that hydrodynamic or shear stress in BioWave® is therefore greatest when the culture bag is used with minimum filling level.

A further finding is the opportunity for power input regulation by medium feeding. In other words, raising culture volume decreases the specific power input and consequently shear stress in BioWave® at constant rocking angle and rate, but impairs its mixing and aeration. Accordingly, periodic medium feeding should be accompanied by an increase in the rocking rate to avoid mass transfer limitations, which adversely affect cell growth and product formation. Up to rocking rates of 20 rpm, the specific power input is always directly proportional to the rocking rate at constant rocking angle and culture volume. If the rocking rate of the culture bag with maximum filling level is further increased, the power input may level out and even be followed by a slight decrease. This phenomenon can be explained by the occurring phase shift of the wave towards rocking movement. Numerous experiments in our lab have confirmed our assumption that, in this case, a subsequent increase in the rocking rate not only lowers hydrodynamic stress for cells, but also improves nutrient and oxygen transfer efficiency, which in turn promotes cell growth [4, 8, 40].

3.3 Reynolds Number and Fluid Flow

A modified Reynolds number (Re_{mod}) was introduced to describe fluid flow in BioWave® [40]. The Reynolds number is generally governed by (3), where w is the fluid velocity, l is the characteristic length of the system (in our case, of the culture bag), and v is the kinematic viscosity of the culture medium. This dimensionless number describes the ratio of internal force to internal friction.

$$Re = \frac{w * l}{v} \tag{3}$$
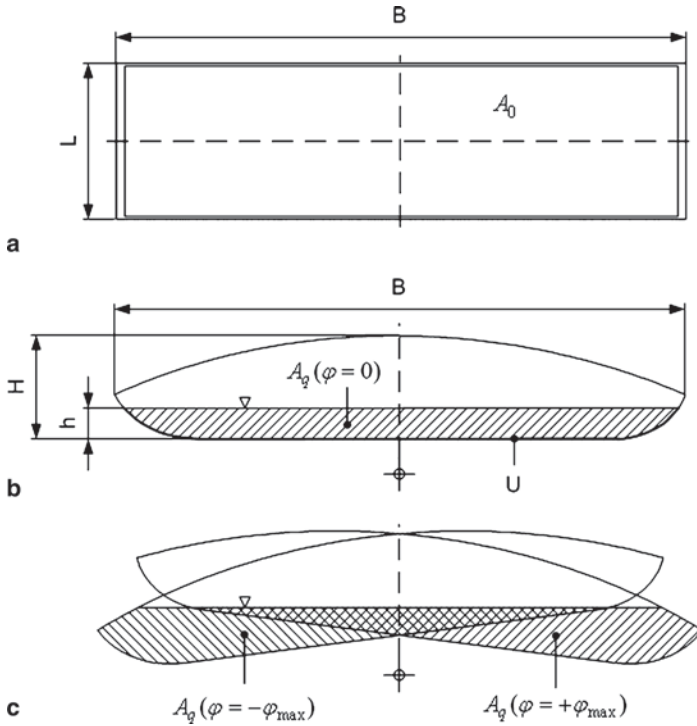
Fig. 5 Assumptions used to estimate Re_{mod} in the BioWave®: (**a**) top view of culture bag, (**b**) cross-section of culture bag in initial position ($\varphi = 0$); (**c**) cross-section of culture bag in final position ($-\varphi_{max}$ and $+\varphi_{max}$)

In order to determine Re_{mod}, the characteristic length (l) can be assumed to be a rectangular cross-section calculated from liquid level (h) and the width of the culture bag (B) preconditioned steady state (Fig. 5b). In this case, the liquid level (h) of the culture bag is a function of the culture volume (V) and the bag geometry (i), given by the ratio of L to B. However, as a result of its shape and fixation, the cross-section of a culture bag deviates from a rectangular cross-section according to filling level and bag type. It is clearly possible to correct such deviations by experimental determination of true length (U) under a fluid's surface area (A_0). For BioWave®'s culture bags, we defined the characteristic length (l) using (4).

$$l = \frac{2 * A_q}{U} \tag{4}$$

Substituting A_q in (4) with and $A_q = h * B$ and U in (4) with $U = 2 * h + B$, we arrive at (5):

$$l = \frac{2 * h * B}{2 * h + B} \tag{5}$$

So far, fluid velocity (w) has been defined as the ratio of medium flow rate or volumetric flow rate $\dot{V}$ to the hydraulic cross-section (A_q). Clearly, the volumetric

flow rate $\dot{v}$ is dependent on the culture bag, namely its type and geometry (i), the culture volume (V), the rocking angle (φ) and the rocking rate (k). Depending on the combination of these factors, the volumetric flow rate varies and as a result differing amounts of substances are exchanged over the rotation point (Fig. 5c). The influence of bag type, culture volume, rocking rate and rocking angle on the volumetric flow rate was determined by experiments and analysed by introducing a correction factor C_1. This factor, obtained with the aid of CAD and regression analysis, differs for every bag type, rocking rate, rocking angle and culture volume [4, 40].

If we assume that not all of the total culture volume of the bag is moving over the rotation point (Fig. 5c) and that the volumetric flow passes the rotation point twice, and w can be calculated with (6) and (7).

$$\dot{V} = V * 2 * k * C_1 \tag{6}$$

$$w = \frac{V * 2 * k * C_1}{A_q} \tag{7}$$

Applying (5), (7) and $A_q = h*B$ to (3) provides a first approach for calculations of modified Reynolds numbers [see (8)]:

$$Re_{mod} = \frac{V * k * 4 * C_1}{v * (2 * h + B)} \tag{8}$$
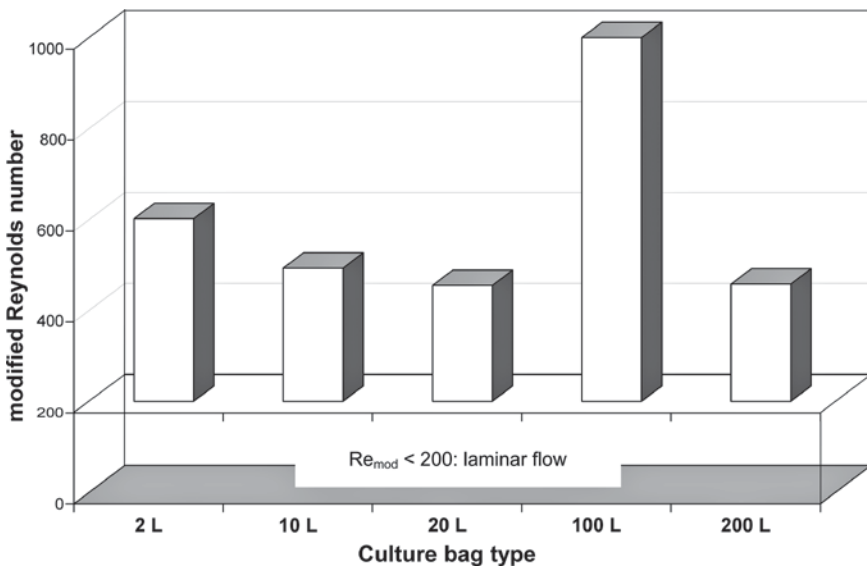
Fig. 6 Transition areas from laminar flow to turbulent flow for different culture bags (filling level 10–50%)

From calculations based on (8) and through visual observation, we determined the areas of transition from laminar flow to turbulent flow for every culture bag type (Fig. 6).

These transition areas vary according to the type of culture bag, while critical values for Re_{mod} range between 200 and 1,000. Only at values of Re_{mod} outside this range does a turbulent flow in the culture bags take place. In order to compare fluid flow in BioWave® with fluid flow in stirred bioreactors, the correction factor C_2 (depending on bag type and describing the correlation of BioWave®'s Re_{mod} and Re_{mod} from stirred bioreactors) was established. C_2 is incorporated into (8) to form (9) as follows:

$$Re_{mod} = \frac{V * k * 4 * C_1 * C_2}{v * (2 * h + B)} \tag{9}$$

In (10) a generalised calculation basis for Re_{mod} is presented where C is used to represent $4*C_1*C*_2$.

$$Re_{mod} = \frac{V * k * C}{v * (2 * h + B)} \tag{10}$$

As shown in Fig. 7a, changing Re_{mod} values at identical process parameters are caused by varying bag geometries. This is clearly attributed to different possibilities for wave development and propagation, and depends on bag dimension as well as specific power input. Only in 2-L culture bag was an almost linear increase in Re_{mod} found. When increased, culture volume results in reduced headspace volume as demonstrated in Fig. 7b: the wave motion is not sufficiently developed and Re_{mod} decreases with increasing culture volume.

Finally, we set out to characterise fluid flow and to determine Reynolds number in BioWave® by using Computational Fluid Dynamics (CFD). Computations with varying rocking rates and rocking angles at different filling levels were made for different culture bag types. Using the Volume of Fluid (VOF) method resulted in the first 2D- and 3D-models correctly describing the free fluid surface. Figures 8, 9, 10 illustrate the first simulation results calculated with Fluent 6.3. Wave development and propagation is already recognisable.

Experimental measurements for validating the computations are in preparation.

3.4 Mixing Time

Mixing time (θ_{95}, defined as time required to achieve 95% homogeneity) was measured by injecting a pH effector (KCl or NaCl) and using the iodine-thiosulfate-decolourisation method. For more detailed information on the mixing-time experiments performed, the reader is referred to [40] and [61]. Values of mixing time were observed to be directly dependent on rocking rate and indirectly dependent on rocking angle in BioWave®. Mixing times achieved with 40% and 50% culture volume ranged between 10 s and 1,400 s for Newtonian fluids [4, 19, 40] and can
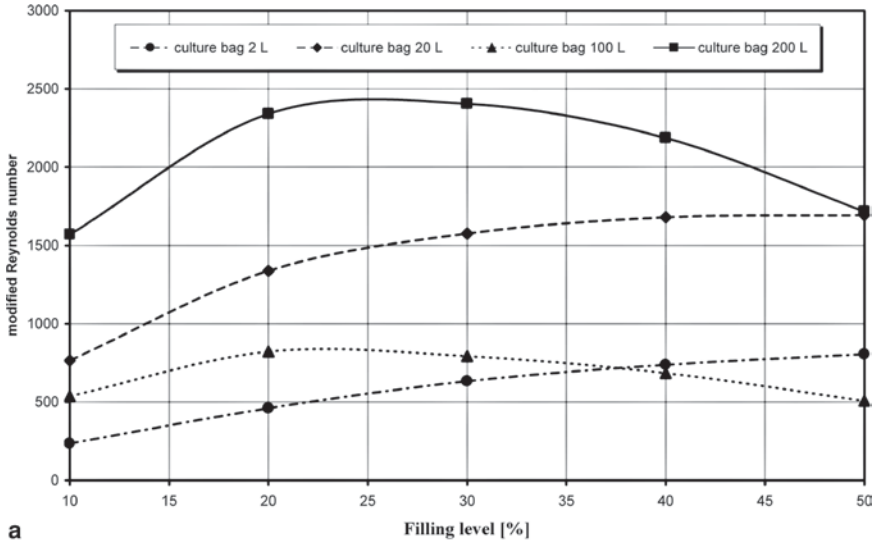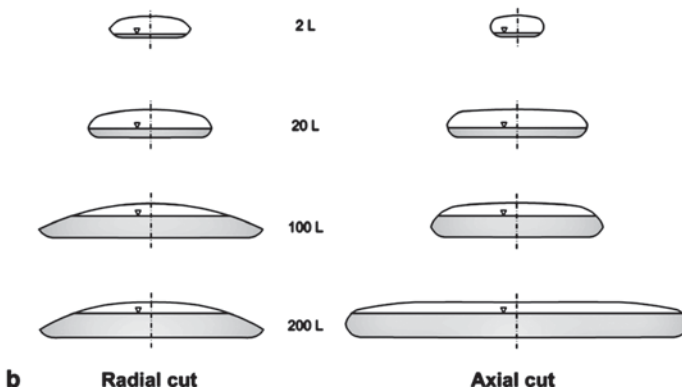
Fig. 7 Modified Reynolds numbers and cross-sections: (**a**) Re_{mod} values for culture bags 2 L, 20 L, 100 L and 200 L operating with constant rocking rate of 18 rpm and rocking angle of 8°; (**b**) cross-sections of different culture bags with 50% filling level

be regarded as satisfactory values for cell-culture bioreactors (Fig. 11). Independent of bag type, the most inefficient mixing takes place at the smallest possible rocking rate and rocking angle with 50% culture volume. Whereas the most efficient mixing was obtained in culture bag 2 L, the most inefficient mixing of all bags investigated was shown by culture bag 20 L. Interestingly, an increase in Re_{mod} over values between 1,000 and 2,000 did not further reduce the mixing time.
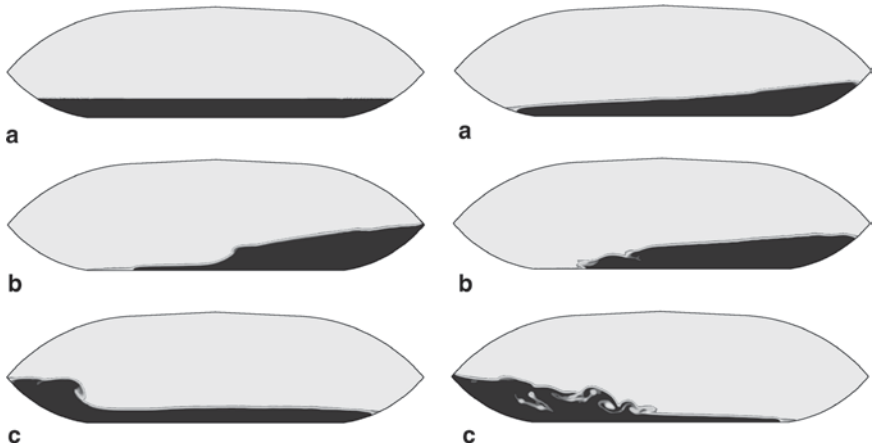
Fig. 8 BioWave®-2D simulation run with Fluent 6.3 at (**a**) 0 s/0°, (**b**) 0.5 s/5.8°, (**c**) 0.9 s/4.24°, (**d**) 1.1 s/1.55°, (**e**) 1.5 s/−4.24° and (**f**) 1.8 s/−6° of real time/angle [water/medium (*black*) and air (*white*)]. The culture bag was filled up to a volume of 400 mL. The simulation was carried out for rocking angle of 6° and rocking rate of 25 rpm (sinus-like movement assumed)
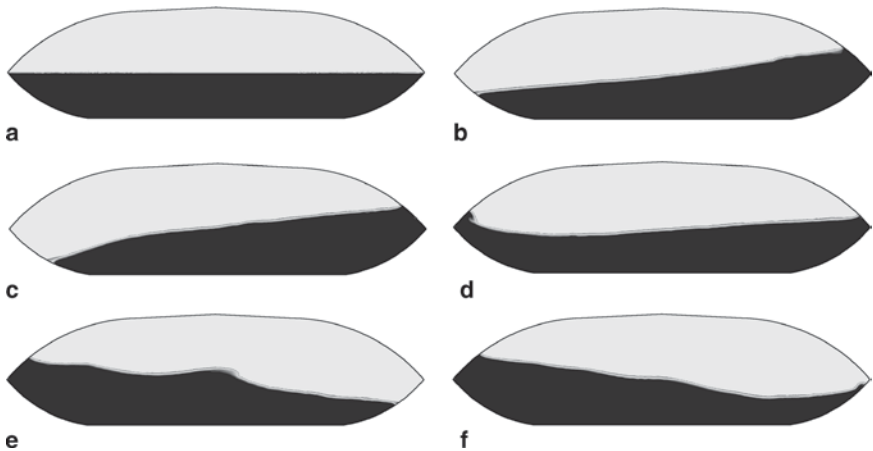
Fig. 9 BioWave®-2D simulation run with Fluent 6.3 at (**a**) 0 s/0°, (**b**) 0.5 s/5.8°, (**c**) 0.9 s/4.24°, (**d**) 1.1 s/1.55°, (**e**) 1.5 s/−4.24° and (**f**) 1.8 s/−6° of real time/angle [water/medium (*black*) and air (*white*)]. The culture bag was filled up to a volume of 1,000 mL. The simulation was carried out for rocking angle of 6° and rocking rate of 25 rpm (sinus-like movement assumed)
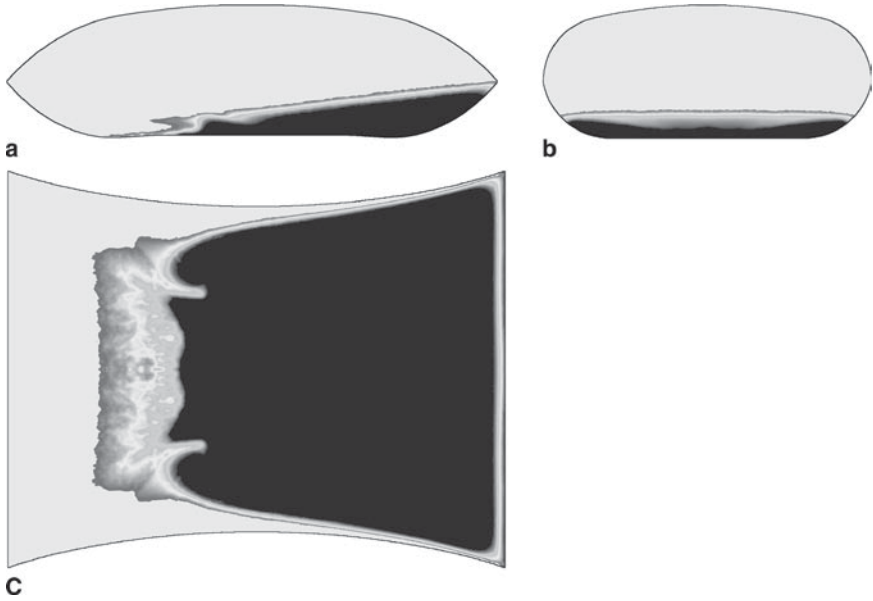
Fig. 10 BioWave®-3D simulation run with Fluent 6.3 at (**a**) longitudinal cut, (**b**) lateral cut and (**c**) top view at 1.3 s/5.34° of real time/angle [water/medium (black) and air (white)]. The culture bag was filled to a volume of 700 mL. The simulation was carried out with a rocking angle of 6° and a rocking rate of 15 rpm using symmetry plane in longitudinal cut (sinus-like movement assumed)

Culture bag	Mixing time [s]
2 L	9 - 264
20 L	40 - 1402
100 L	22 - 837
200 L	65 - 874
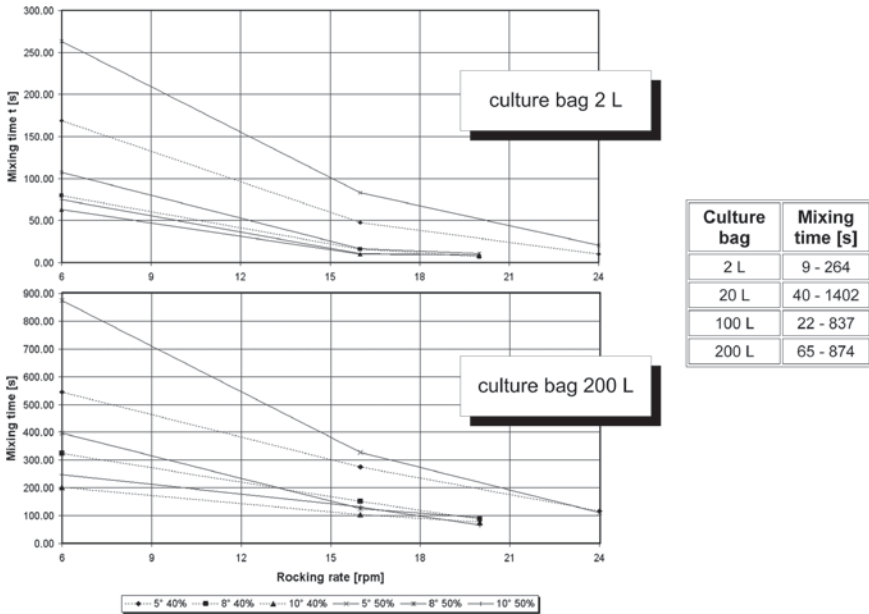
Fig. 11 Mixing times in BioWave® 20 SPS and 200 SPS operating with culture bag 2 L, 200 L and 40% as well as 50% filling level

3.5   Residence Time Distribution

Residence time is an important value with which to characterise continuously operating bioreactors and enables a comparison of real bioreactors with ideal bioreactors. From our step experiments (Fig. 12b) using the displacement technique and NaCl as tracer [40, 61], it is evident that BioWave® operating in continuous perfusion mode (culture bag 2 L, 50% filling level, 5.1°, 6 rpm, τ of 2.6 h) can be described by the ideally mixed stirred tank reactor model. Figure 12a compares the response measured in BioWave® and the residence-time distribution in an ideally mixed stirred tank reactor. Both curves show an identical course and are congruent [4].
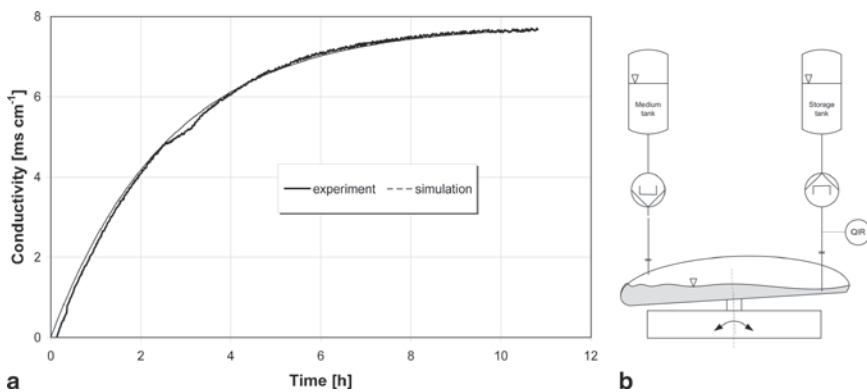
Fig. 12 Residence time distribution: (**a**) measured in BioWave® and theoretical residence time distribution of an ideally mixed stirred tank reactor; (**b**) set-up for experimental determination of BioWave®'s residence time distribution

3.6   Scale-Up

Scale-up of the culture volume from laboratory to pilot scale (1:5 or 1:10 steps) in BioWave® systems is usually realised by carrying out a number of experiments, the so-called trial and error principle. Achieving the constant process intensity desired, and therefore the success of the scale-up, depends partly on luck as well as on the cultivation experience of the person growing the cells and their knowledge of the bioreactor. Well-investigated scientific scale-up criteria which are mainly used for stirred bioreactors (namely critical tip speed, specific power input, geometry, oxygen transfer and fluid flow) are not described for wave-mixed bioreactors.

   For cell cultures, the expertise in our working group on engineering aspects of BioWave® and the amount of data at our disposal have allowed us to realise the scale-up from BioWave® 20 SPS with 1 and 10-L culture volume to BioWave® 200 SPS with 50 and 100-L culture volume while guaranteeing similar fluid flow

conditions and an optimum oxygen supply independent of scale. For this reason, we decided to use a modified Reynolds number together with our knowledge of the influence of aeration rate, rocking angle, rocking rate and culture volume on oxygen transfer efficiency as scale-up criteria for BioWave® cultivations in a first approach. For the next scale, we were already in a position to set up values for the filling level, rocking rate and rocking angle corresponding to the optimum Re_{mod} range which had been found in the optimisation experiments in 2-L bags.

In the future, it is expected that 2D and 3D CFD models characterising the local velocity and concentration profiles in BioWave® will support its scientific scale-up (see also Sect. 3.3). Such models will allow comparison with CFD models from stirred bioreactors, and thus fluid flow-based comparison of BioWave® and stirred bioreactors. In summary, CFD modelling will contribute to optimising bag design, to defining scale-up criteria, to comparing different bioreactor systems, as well as to developing processes with optimum growth and production.

4   Biowave® Applications

Previously outlined findings allow us to conclude that BioWave® is most suitable for small- to middle-scale processes with animal cells, plant cells and microbial cells with low oxygen demands. These production organisms are used to generate expanded functional cells for process inoculum and patient-specific therapies, recombinant therapeutic proteins (which also include antibodies), viruses for vaccines and gene therapies, secondary metabolites and niche products such as immunomodulators, chiral building blocks and biological pesticides. As already described in Sect. 3.1, optimised high cell-density cultivations of bacteria or yeast strains cannot be accomplished in BioWave® without modifications to the standard cultivation bag, and aeration with pure oxygen or aeration by an external air pump.

With regard to the efficient BioWave® applications to be outlined in the following sections, it is essential to realise the upstream cultivation steps in single-use systems (e.g. t-flasks, Integra's CeLLine, Corning®HYPERFlask, shake flasks, Sartorius Stedim's SuperSpinner or Nunc CellFactory). Furthermore, a reduction in intermediate cultivation steps warrants investigation. This is the case, for instance, when the mechanically driven culture bag of BioWave® is inoculated with pooled suspension cells from t-flasks or thawed cells directly from a static bag (e.g. CryoBag, www.origen.com), where the cells have been cryogenically preserved in liquid nitrogen.

4.1   Seed Inoculum Production with Animal Cell Cultures

The original idea was to replace animal suspension cell-based seed inoculum production in spinner flasks (which involves numerous manual operations) and in stirred bioreactors made of glass or stainless steel with the first wave-mixed laboratory

bag bioreactor type, Wave Bioreactor. Because of its easy handling, low contamination rates below 1%, and the cell amounts achieved (normally ranging between 2×10^6 cells mL^{-1} and high cell densities) at viabilities $\geq 95\%$ [8, 19], it is not surprising that BioWave® systems have already been accepted as approved bioreactors for seed inoculum train and seed train production in R&D processes and in GMP-compliant biomanufacturing. A protocol for rapid, successful CHO suspension cell expansion in a chemically defined minimal culture medium and a 2-L culture bag of a BioWave® operating in feeding mode is presented in [39]. From our experience, this method can also be applied with minor modifications to other animal suspension cells, such as insect cells and human embryogenic kidney cells. When using the recently introduced CultiBag (www.sartorius-stedim.com), the bag rinsing step described for growing animal suspension cells in chemically defined minimal culture medium can be omitted [63, 64].

4.2 Therapeutic r-Protein and Antibody Secretion in Animal Cell Cultivation

Because animal cell-derived therapeutic r-proteins (e.g. erythropoietin for treatment of anaemia or tPA, also called Activase®, for ischemic stroke patients) and monoclonal antibodies (e.g. Avastin® for metastatic colorectal cancer treatment or Herceptin®, trastuzumab, for HER 2-protein overexpressing metastatic breast cancer patients) are modern biotechnological products with tremendous growth potential and therefore attract special industrial interest, great international efforts have been made to shorten their development time and reduce their development costs. By using a wave-mixed bioreactor instead of a stirred bioreactor in animal cell cultivations, cost savings can result from shortened set-up and production turnaround times, disposing with sterilisation and cleaning steps, and a reduction in contamination risk and validation steps [65, 66]. For BioWave®, production protocols for the CHO suspension cell-based model protein SEAP [39, 65, 66], the HEK suspension cell-secreted Resistin [8] and insect cell-expressed proteins are available [12, 14, 59].

In order to secrete SEAP (human placental secreted alkaline phosphatase), an engineered CHO suspension cell line [67] was cultivated. The clone CHO XM 111–10 (obtained from Prof. Dr. Martin Fussenegger, Swiss Federal Institute of Technology in Zurich) contains the tetracycline-promoter PhCMV-1, which controls SEAP expression. Cell growth behaviour and glycoprotein production in BioWave® experiments could therefore be regulated by the addition or withdrawal of tetracycline. For this purpose, a two-stage process with a 4–5-day average growth phase (feeding mode, mixture of chemically defined CHOMaster HP-1 and HP-5 minimal medium with tetracycline, Cell Culture Technologies) and a 14-day average production phase (batch-mode, CHOMaster HP-5 medium without tetracycline, Cell Culture Technologies) after medium exchange was accomplished up to 10-L culture volume [8, 65]. For the evaluation of maximum cell amounts and

SEAP activities achieved in BioWave®, further experiments in disposable membrane bioreactors (high cell-density systems: CeLLine CL 1000, MiniPerm operating with classic kit production module, Cell-Pharm 100 with BR 130 module) and a bubble-free aerated stirred bioreactor (2 L BIOSTAT B) were carried out, and finally a costing based on 1,000 Units SEAP was done. Whereas all disposable systems were inoculated with pooled cells from t-flasks, the inoculum for the stirred cell-culture bioreactor made from glass was always traditionally produced in spinner flasks. As expected, the high cell-density systems guaranteed maximum living cell counts, exceeding ten million cells per mL. The maximum living cell count was 5.4×10^7 cells mL^{-1} and was provided by Sartorius Stedim's MiniPerm system, although the small culture volume (25 mL) of this system allows five-times lower SEAP activities than BioWave®, which has the maximum total product output. Indeed, BioWave® generated middle cell densities (5×10^6 cells mL^{-1}) associated with 20% higher SEAP activities (5,160 Units SEAP) in contrast to the stirred cell-culture bioreactor, where 3×10^6 cells mL^{-1} and 4,120 Units SEAP were reached on average. In addition to reducing manual cultivation steps, investment in manpower and material used, this also explains the possible 50% saving in process costs when producing SEAP in BioWave® in place of BIOSTAT B [39, 68]. A further increase in SEAP activity [8] is realisable with a simple shift of cultivation temperature from 37 to 30°C, which causes a growth arrest in the G1 phase of the cell cycle according to Kaufmann et al. [69].

The quasi-stable HEK-293 EBNA suspension cell line [8], which produces Resistin, was established by Cytos Biotechnology and was based on the company's pCyTS™ system. The cells were routinely maintained in serum-free medium and t-flasks at 37°C (CO_2 incubator). BioWave® inoculation and its operation while proliferation (growth phase) occurred in the same manner as exemplified for the CHO suspension cells (with the exception of the culture medium, which was chemically defined InVitrus VP-6 medium from Cell Culture Technologies or CD 293 AGT medium from Gibco/Invitrogen) for 1-L, 10-L and 50-L culture volume. Extracellular Resistin formation (production phase of approximately 7 days) was affected by a temperature shift to 29°C. Cell amounts of 3×10^6 cells mL^{-1} delivered 1.25 g of Resistin in 50-L chemically defined InVitrus VP-6 medium [70]. Up to 45% higher maximum cell amounts were measured in cultivations with the CD 293 AGT medium in BioWave®'s culture bag for 1- and 10-L culture volume [71].

Insect cells (especially *Sf*9- and *Sf*21 suspension cells) used with the baculovirus expression vector (BEV) system and wave-mixed bioreactors are regarded as fast, efficient and easy-to-handle systems for the manufacture of r-tool proteins. Within a few days, desired r-proteins can be produced in mg- to g-range in serum-free culture medium. Again, biphasic cultivation with decoupled growth and r-protein production, proceeding after virus infection, becomes necessary. Mass transfer limitations are normally prevented by stepwise rocking rate increase at a constant rocking angle [12] or rising rocking rate and angle [14]. Moreover, the increased oxygen demand in the production phase can be met by aeration with pure oxygen [72]. The report of Schlaeppi et al. [12] describes the efficiency of BioWave® in a semi-automated large-scale process for the production of four tagged proteins in a

BEV/*Sf*21 cell culture. In less than 24 h, final yields between 1 and 100 mg at purities between 50 and 95% were processed in up to eight BioWave® systems, with 10-L culture volume per bioreactor.

4.3   Animal Cell-Based Virus Production

European approval of the BEV/insect cell culture system-based Cervarix® (vaccine against cervical cancer from Glaxo SmithKline) in autumn 2007 started a new wave in the development of biologics from insect cell lines. It therefore comes as no surprise that further research activities are aimed at animal cell-based virus production for vaccine manufacturing and gene therapies, and especially the possible replacement of traditional vaccine production bioreactors (roller flasks, Cell Factories, stirred microcarrier bioreactors) by BioWave®.

Slivac et al. [13] developed the biphasic production of Aujeszky's disease virus (ADV) vaccine for BioWave® with 450-mL culture volume. BHK 21 C13 suspension cells which had been previously cultivated in spinner flasks were grown (5.5×10^5 cells mL^{-1}, 6°, 10 rpm, 0.44 vvm) during 3 days up to a maximum cell amount of 1.82×10^6 cells mL^{-1} at viabilities of 99%. Optimum growth conditions were ensured by a partial medium exchange of 65% on the second day of the growth phase. 144 h after infection with gE⁻ Bartha K-61 strain virus suspension ($10^{5.9}$ TCID$_{50}$, MOI of 0.01), 400 mL of ADV harvest characterised by a titre of $10^{7.0}$ TCID$_{50}$ mL^{-1} was achieved. This means that 40,000 doses of pig vaccine can be prepared.

As demonstrated by Genzel et al. [9, 10] and Dietzsch et al. [73], MDCK cells and Vero cells can be advantageously grown on Cytodex™1 microcarriers (2 g L^{-1}, 4 g L^{-1}) and human as well as veterinary influenza viruses can be successfully secreted into serum-supported culture medium in BioWave® with 1-L culture volume. Microcarrier transfer, cell attachment, cell proliferation, washing, medium exchange, and virus infection were realised using methods described by Genzel et al. [10] on cocker spaniel cells, which form host cells for equine influenza virus replication. Remarkably, the final cell amount on the microcarriers in BioWave® was about double that of a similarly investigated stirred bioreactor for the same application. 20-h post infection peak equine influenza virus titres of $10^{7.7}$ TCID$_{50}$ mL^{-1} were reached [10].

The possibility of transferring a registered roller flask-based manufacturing process for a mink enteritis virus (MEV) vaccine to a BioWave® was investigated by Hundt et al. [11]. After 5-days preculture of feline lung fibroblasts (E-FL) in roller flasks, the cell-microcarrier mixture (2×10^5 cells mL^{-1} and 2 g L^{-1} Cytodex™1 microcarriers) was transferred to 2-L and 20-L culture bags containing 1 L and 10 L of serum-supported culture medium, respectively. The BioWave®-MEV vaccine-production process, which included three to four medium exchanges and virus harvests, culminated in virus titres between $10^{6.6}$ and $10^{6.8}$ TCID$_{50}$ mL^{-1}. These documented values are approximately ten-times higher than those of the roller flask process.

4.4 Plant Cell-Based Bioprocessing: Production of Biomass and Secondary Metabolites

For more than 45 years researchers have focused on in vitro production of plant-derived biologically active substances, which include secondary metabolites and r-proteins and are significant in the pharmaceutical industry, the food industry and cosmetics. Instead of growing whole plants, the cultivation of plant cell and tissue cultures in bioreactors guarantees well-controlled process conditions with consistent product quantity and quality, and also simplified process validation. Prominent commercial product examples are shikonin (antimicrobial rich reddish-purple pigment for lipsticks), ginsenosides (food additives and whitening substances), paclitaxel (anti-cancer drug) and the first plant cell-produced veterinary vaccine (Newcastle Disease Virus) [74–77].

In commercial production processes, plant cell suspensions represent the most often used plant cell culture type, although they tend to genetic instability over time [78, 79]. Despite their special morphological character and resulting high sensitivity to changes in culture environment and shear stress, hairy root cultures (generated by the transformation of explants with agropine and mannopine type strains of *Agrobacterium rhizogenes*) are attractive alternatives to the dominating plant cell suspensions. Hairy roots synthesise secondary metabolites at similar or higher levels to those found in whole plants [80], possess r-protein expression ability [81], show comparable doubling times to plant cell suspensions [78, 82], and are genetically and biochemically long-term stable [1]. Because their auxin metabolism is altered, the addition of exogenous growth regulators to the culture medium is unnecessary. Nevertheless, it is more difficult to grow hairy roots in bioreactors without mass transfer limitations and root damage than plant suspension cells.

4.4.1 Cultivation of Plant Cell Suspensions

Plant cell suspensions differ from animal suspension cells mainly in cell size (10–100 μm), cell shape (spherical and rod morphology), cell aggregation rate (high), doubling time (between 0.6 and 5 days) and product formation (often intracellular). Furthermore, their optimum growth requires a lower cultivation temperature (25–27°C) at a wider pH range (5–7) with aeration rates (0.1–0.3 vvm) and tip speeds (≤ 2.5 m s^{-1}) being comparable to those of animal suspension cells. However, it should be borne in mind that in plant cell culture broths with high cell concentrations, non-Newtonian flows associated with higher culture viscosity are available [83, 84]. Thus, the cultivation of well-growing plant cell suspensions is subject to mass transfer limitations to a greater extent than those of animal cell suspensions, which always behave like a Newtonian fluid. Indeed, the same bioreactor types (e.g. stirred bioreactor, bubble column, airlift bioreactor), which are the most suitable for cultivating animal suspension cells (with bubble-aeration), are successfully used for processes with plant cell suspensions [5]. When considering

bioreactors for plant cell cultivations, BioWave® is the most-cited disposable system for suspension cultures. Table 3 gives a résumé of reported plant cell suspension experiments conducted in this bioreactor type in batch and fed-batch mode. In order to avoid mass transfer limitations, the rocking rate was simultaneously increased with cell broth viscosity in the culture bag. For all cultivations, special culture bags with screw caps were utilised to facilitate easy inoculation and sampling [4].

The biomass production results presented reveal the potential of BioWave® for growing tobacco, grape and apple suspension cells up to 10-L culture volume. Maximum biomass productivities of 40 g fresh weight L^{-1} d^{-1} with excellent maximum doubling times of 2 days and low shearing (indicated by low specific power input, cell viabilities ≥95% and no significant change in cell morphology, growth and metabolism over long cultivation periods) were observed [5]. In our experience, the addition of cell protection agents (e.g. Pluronic F 68) is completely unnecessary in BioWave®. Because of reduced foaming as well as the absence of air bubbles and wall growth phenomena known from fast-growing plant cell suspensions in traditional bubble-aerated stirred and pneumatically driven bioreactors (bubble column, airlift bioreactor) [86], antifoam agents (e.g. Antifoam C) do not need to be added.

This will simplify the expansion of active plant cell biomass (non-engineered cells), which has recently been done in connection with natural cosmetic ingredients in BioWave® [87]. The activities of Griehl et al. [88], which focussed on the establishment and in vitro cultivation of marine macrophytic cell cultures of

Table 3 Summarised results of plant cell suspension cultivation in BioWave®

Product	Plant species	Culture volume (L)	Process mode	Maximum product yield	References
Biomass	*N. tabacum* (BY-2[a])	1	Batch	26 g fw L^{-1} d^{-1}	[85]
	N. tabacum (T 408)	1	Batch	16 g fw L^{-1} d^{-1}	[85]
	N. tabacum L.	10	Batch	22 g fw L^{-1} d^{-1}	[4, 5]
	V. vinifera	1	Batch[b]	40 g fw L^{-1} d^{-1}	[5, 6]
	M. domestica	1	Batch	35 g fw L^{-1} d^{-1}	[5]
Taxanes	*T. baccata*	0.4	Fed-batch[c–f]	10 mg L^{-1} paclitaxel 5 mg L^{-1} baccatin III	[3, 5, 7]
			Fed-batch[c,e–g]	20.8 mg L^{-1} paclitaxel 7.8 mg L^{-1} baccwatin III	

[a] Wildtype
[b] UV-B irradiation
[c] Feeding during growth phase
[d] Free cells
[e] Elicitation by methyljasmonate
[f] Additional precursor feeding (mevalonate, *N*-benzoylglycine)
[g] Immobilised cells

Ulva lactuca (not included in Table 3), tend in the same direction. In this case, BioWave® simulates the natural environment of macroalgae, which are typically field-grown for commercially available products.

It is also worth mentioning that the paclitaxel productivity depicted in Table 3 for BioWave® running with immobilised Taxus suspension cells is one of the highest reported by academic researchers to date [3, 5, 7].

4.4.2 Hairy Roots as Production Organisms

Again, BioWave® can be regarded as the prevailing disposable bioreactor type for hairy root cultivations. In contrast to submerged plant cell suspension experiments, hairy roots normally prefer emerged cultivation conditions (realised in gas-phase bioreactors) and ebb-and-flow, also called temporary immersion, conditions. For secondary metabolite expression investigations, two transformed root lines were cultivated in ebb-and-flow mode in BioWave® (25°C, 6°, 6 rpm, 0.15–0.3 vvm) for 28 and 56 days. The culture medium was fed with increasing root amount, but the rocking angle and rocking rate were maintained constant. In this way, the roots were temporarily immersed in the culture medium. Hairy roots of *Hyoscyamus muticus* (clone KB5, light-culture), supplied by Dr. Kirsi-Marja Oksman-Caldentey, VTT Espoo, Finland, produce intracellular hyoscyamine in Gamborg's B5 medium without phytohormones [89]. *Panax ginseng* hairy roots (clone T12, dark-culture, established by Anna Mallol at the University of Barcelona, Spain) growing in chemically defined SH medium intracellularly express ginsenosides [2]. Prolongation of cultivation time could be achieved by additional medium exchange every 14 days. Whereas BioWave® with *Panax ginseng* hairy roots was inoculated with 10 g fw L^{-1}, the inoculation rate for *Hyoscyamus muticus* hairy roots amounted to 5 g fw L^{-1}. Illumination of the henbane root culture was effected by external fluorescent lamps (Osram L36W72–965 Biolux lamps, 16 h on/8 h off). In the 2-L culture bag (0.1-L initial culture volume and 0.5-L final culture volume), uniform distribution of proliferating henbane and ginseng hairy roots was observed. Maximum biomass productivities (5.1 g fw L^{-1} d^{-1} for *P. ginseng*, 20.3 g fw L^{-1} d^{-1} for *H. muticus*) and product yields (146 mg g^{-1} dw ginsenosides, 5 mg g^{-1} dw hyoscyamine) achieved in BioWave® operating with the 2-L culture bag were two to three times higher than in glass spray reactors for both root clones [2, 4, 5, 8]. In the 20-L culture bag (1-L initial culture volume and 5-L final culture volume), approximately 3.6 lower biomass productivities were guaranteed and the propagated biomass was localised at three points. The uniformity of root tissue distribution in BioWave®'s culture bag seems to be the key issue for the minimisation of mass transfer limitations and therefore optimum root growth. For this reason, culture bags with integrated mesh supporting root immobilisation were developed for culture volumes above 0.5 L. Finally, for *H. procumbens* (grapple plant) hairy root mass propagation, the proof of concept was done by our group in BioWave® with the 2-L culture bag (0.1-L initial culture volume and 0.5-L final culture volume). 154 g (fw) root biomass was harvested from two culture bags after 28 days [90].

4.5 Microbial Immunomodulator Secernation

For the first time, a microbial immunomodulator secernation in BioWave® was established and scaled-up to pilot scale. A facultative anaerobic *E. coli* strain secreting an immunologically active substance for chronic intestinal inflammatory conditions, allergies and cancer was grown for this purpose. A hybrid production facility consisting of three BioWave® 200 SPS systems (Fig. 13) was set up and tested. During batch runs at 37°C, the BioWave® systems were operated with synthetic cultivation medium and specially designed culture bags (120-L culture volume), which had been previously inoculated with cells from gas permeable plastic bags instead of the usual shake flasks.

The runs provided cell densities from 7×10^8 cells mL^{-1} to 1×10^9 cells mL^{-1} and product concentrations between 30 mg L^{-1} and 40 mg L^{-1}. In four GMP manufacturing experiments performed in the clean rooms of the company Laves-Arzneimittel (Switzerland), we were able to actively secrete the main substances responsible for inhibition of T-lymphocyte proliferation into BioWave®'s culture bag [8, 40].

4.6 Production of Chiral Building Blocks and Biological Insecticides by in Vitro Cultivation of Fungal Species

There are two further approaches to microbial cultivations of eukaryotic fungal species in BioWave® [91–94]. Jablonski-Lorin et al. [91] show the capability and advantages of disposable wave-mixed bioreactors for large-scale biotransformations.
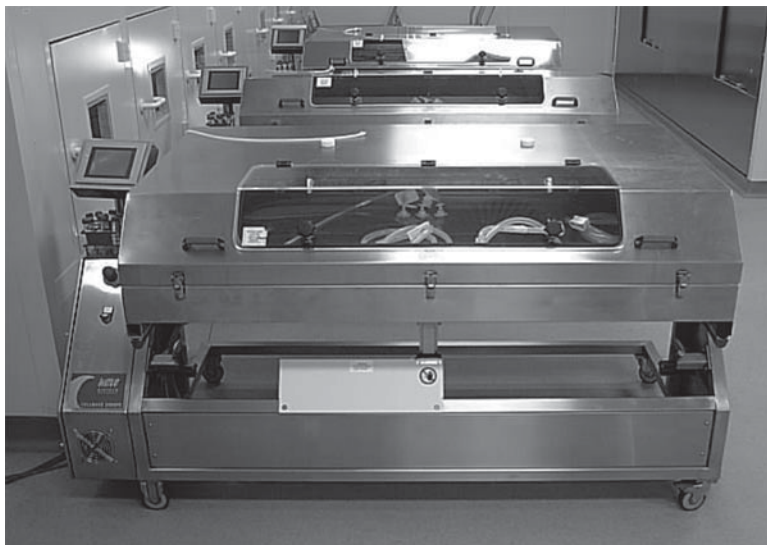
Fig. 13 BioWave® 200 SPS systems used in GMP manufacturing experiments (photograph provided by Laves-Arzneimittel)

The highly stereoselective bioreduction of ethyl cyclohexanone-2-carboxylate (1) with *Schizosaccharomyces pombe* was established and optimised for BioWave® operating with 1-L, 8-L and 80-L culture volume. In this way, the pure chiral building block Ethyl (1S, 2S)-*trans*-2-hydroxycyclohexane carboxylate (2), representing an important intermediate step in pharmaceutical drug synthesis, was obtained in 56% yield with a diastereomeric ratio (dr) of 99:1 and an enantiomeric ratio (er) of 99.5:0.5 after rectification. The biotransformation extended over 44 h and was achieved without the addition of antifoaming agent, which has to be stringently used in the classical process. Consequently, the subsequent product purification could be simplified for the BioWave® process.

Canales et al. [93] and Hess et al. [92, 94] demonstrated the laboratory BioWave®'s ability for mass production of filamentously growing fungus *Erynia neoaphidis*, which is useful for attacking a wide range of aphid species in Europe. The dark cultivations (20°C, 8°, 25 rpm, 1.5–3 vvm, YEPG medium, standard 2-L culture bags) resulted in a mean biomass production rate of 7-g dry weight per day [93] and encouraged the researchers to scale-up the process for the standard 20-L culture bag [94]. However, it should be mentioned that in all the experiments the stability of the cultivation temperature was ensured by operation of BioWave® in an air-conditioned cabinet and the high aeration rate required an external air pump.

4.7   In Vitro Production of Insecticidal Nematodes in Liquid Medium

The same working group discovered the suitability of BioWave® for in vitro production of two nematode species: *Steinernema feltiae*, used in pest control of Sciaridae (dark-winged fungus gnats) larvae, and *Heterorhabditis megidis*, which controls larva development of the *Otiorhynchus sulcatus* (black vine weevil) [95, 96]. The final yields of *Heterorhabditis megidis*, which constitute a maximum of 2×10^5 nematodes mL^{-1}, deserve a particular mention. This corresponds to an 80-fold increase in the number of nematodes measured in the inoculum and is the highest value recorded for nematode liquid cultures in bioreactors so far.

5   Conclusion and Outlook

This review shows the popularity of the disposable BioWave®, which is mechanically driven and surface-aerated. Its mass (nutrient and oxygen) and energy transfer, which influence growth and product formation efficiency, are highly dependent on wave development and propagation, which occurs in the culture bag (containing medium and cells). Moreover, the wave induced in the culture bag differs when varying bag scale and dimension, or adjusting the rocking rate, rocking angle and filling level. Determined Re_{mod} numbers, fluid flow, mixing times, residence time

distribution data, volumetric oxygen mass transfer coefficients and values for specific power input evidence BioWave®'s comparability (and sometimes superiority) to surface or bubble-free aerated stirred cell-culture bioreactors made of glass or stainless steel. Furthermore, BioWave®'s suitability for small- and middle-scale processes based on cells with low oxygen demands is obvious.

Results from animal cell cultivations aimed at cell expansion, secretion of therapeutic r-proteins (including antibodies) and viruses, and results from plant cell suspension as well as hairy root cultivations for biomass and secondary metabolite production demonstrably support this assumption. This also applies in the case of facultative anaerobic growing *E. coli* strains, selected eukaryotic fungal species (e.g. *S. pombe*, *E. neoaphidis*) and nematodes (e.g. *S. feltiae*, *H. megidis*) generating niche products such as immunomodulators, chiral building blocks and biological pesticides in BioWave®. In order to extend the already wide usage of BioWave® to high cell-density cultivations with bacteria or yeasts, culture bag design modifications need to be made.

When considering advances in personalised medicine, where ex vivo produced functional cells in clinically relevant numbers for cancer-, immuno- or tissue therapies are required, we see an additional application field with a promising future for BioWave®. It is expected that the increase in use of wave-mixed bag bioreactors will continue in spite of the continuing availability of the first stirred bag bioreactors, which are preferred by supporters of traditional stirred bioreactor technology. Finally, we would like to point out that all data presented and applications for BioWave® can be directly transferred to Wave Bioreactor™ and BIOSTAT® CultiBagRM if the bag type (dimension, material) is identical to the examples we have given.

References

1. Eibl R, Eibl D (2002) Bioreactors for plant cell and tissue cultures. In: Oksman-Caldentey KM, Barz WH (eds.) Plant biotechnology and transgenic plants. Marcel Dekker, New York, p. 163
2. Palazón J, Mallol A, Eibl R, Lettenbauer C, Cusidó RM Piñol MT (2003) Planta Med 69:344
3. Bentebibel S, Moyano E, Palazón J, Cusidó RM, Bonfill M, Eibl R, Piñol MT (2005) Biotechnol Bioeng 89:647
4. Eibl R, Eibl D (2006) Design and use of the Wave Bioreactor for plant cell culture. In: Dutta Gupta S, Ibaraki Y (eds.) Plant tissue culture engineering, series: focus on biotechnology, vol 6. Springer, Dordrecht, p. 203
5. Eibl R, Eibl D (2007) Phytochem Rev, DOI 10.1007/s11101-007-9083-z
6. Cuperus S, Eibl R, Hühn T, Amado R (2007) BioForum Europe 6:2
7. Bonfill M, Bentebibel S, Moyano E, Palazón J, Cusidó RM, Eibl R, Piñol MT (2007) Biol Plant 51:647
8. Eibl R, Eibl D (2006) Disposable bioreactors for pharmaceutical research and manufacturing. Proceedings second international conference on bioreactor technology in cell, tissue culture and biomedical applications. Saariselkä, Finland
9. Genzel Y, Behrendt I, Koenig S, Sann H, Reichl U (2004) Vaccine 22:2202
10. Genzel Y, Olmer RM, Schaefer B, Reichl U (2006) Vaccine 24:6074
11. Hundt B, Best C, Schlawin N, Kassner H, Genzel Y, Reichl U (2007) Vaccine 25:3987
12. Schlaeppi JM, Henke M, Mahnke M, Hartmann S, Schmitz R, Pouliquen Y, Kerins B, Weber E, Kolbinger F, Kocher HP (2006) Protein Expres Purif 50:185

13. Slivac I, Srček VG, Radoševic K, Kmetič I, Kniewald Z (2006) J Biosci 3:363
14. Weber W, Weber E, Geisse S, Memmert K (2002) Cytotechnology 38:77
15. Mikola M, Seto J, Amanullah A (2007) Bioprocess Biosyst Eng 30:231
16. Laderman K, Quezada V, Dunphy N, Anderson J, Derecho J, McMahom R, Hsu D, Couture L (2007) DNA production in the Wave Bioreactor under cGMP conditions. http://www.wave-biotech.com/pdfs/press/pDNA_Poster_COH2007.pfd. Accessed 06 November 2007
17. Amanullah A, Burden E, Jug-Dujakovic M, Mikola M, Pearre C, Herber W (2004) Development of a large-scale cell bank in cryobags for the production of biologics. http://www.wavebiotech.com/pdfs/literature/Merck_Cancun-2004.pfd. Accessed 04 November 2007
18. Cronin CN, Lim KB, Rogers J (2007) Protein Sci 16:2023
19. Singh V (1999) Cytotechnology 30:149
20. Ohashi R, Singh V, Hammel JF (2001) Perfusion cell culture in disposable bioreactors. 17th ESACT meeting June 2001. Tylösand, Sweden
21. Tang YJ, Ohashi R, Hamel JP (2007) Biotechnol Prog 23:255
22. Fries S, Glazomitsky K, Woods A, Forrest G, Hsu A, Olewinski R, Robinson D, Chartrain M (2005) BioProcess Int 3:36
23. Negrete A, Kotin RM (2007) J Virol 145:155
24. Rios M (2006) PharmaTech 4:1
25. Pierce LN, Sabraham PW (2004) BioProc J 3:51
26. Hami LS, Chana H, Yuan V, Craig S (2003) BioProc J 2:23
27. Hamis LS, Green C, Leshinsky N, Markham E, Miller K, Craig S (2004) Cytotherapy 6:554
28. Levine B (2007) Making waves in cell therapy: the Wave bioreactor for the generation of adherent and non-adherent cells for clinical use. http://www.wavebiotech.com/pdf/literature/ISCT_2007_Levine_Final.pdf. Accessed 04 November 2007
29. Matthews T, Wolk B (2005) The use of disposable technologies in antibody manufacturing processes. http://www.wavebiotech.com/pdfs/literature/IBCDisposables_2005.pdf. Accessed 04 November 2007
30. Hallmann S, Bertelsen HP, Scheffler U, Luttmann R (2007) Einsatz von Massflow-Controllern zur Steuerung von Bioreaktionsprozessen. Biotechnica October 2007. Hannover, Germany (poster)
31. Morrow KJ (2006) GEN 26:42
32. Houtzager E, van der Linden R, de Roo G, Huurman S, Priem P, Sijmons C (2005) BioProcess Int 3:60
33. CeLLution Biotech BV (2007) Mass transfer in the CELL-tainer® disposable bioreactor. http://www.cellutionbiotech.com. Accessed 20 October 2007
34. CeLLution Biotech BV (2007) Cultivation of PER.C6®-cells in the CELL-tainer® disposable bioreactor. www.cellutionbiotech.com. Accessed 20 October 2007
35. CeLLution Biotech BV (2007) Cultivation of CHO-cells in the CELL-tainer® disposable bioreactor. www.cellutionbiotech.com. Cited October 20, 2007
36. Terrier B, Courtois D, Hénault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Pétiard V (2006) Biotechnol Bioeng 96:914
37. Girard LS, Fabis MJ, Bastin M, Courtois D, Pétiard V, Koprowski H (2006) Biochem Biophys Res Commun 345:602
38. Kilani J, Lebeault JM (2007) Appl Microbiol Biotechnol 74:324
39. Eibl R, Eibl D (2007) Disposable bioreactors for inoculum production and protein expression. In: Pörtner R (ed.) Animal cell biotechnology: methods and protocols. Humana Press, Totowa, NJ, p. 321
40. Eibl R, Eibl D, Pechmann G, Ducommun C, Lisica L, Lisica S, Blum P, Schär M, Wolfram L, Rhiel M, Emmerling M, Röll M, Lettenbauer C, Rothmaier M, Flükiger M (2003) Produktion pharmazeutischer Wirkstoffe in disposable Systemen bis zum 100 L Massstab, Teil 1. KTI-Projekt 5844.2 FHS, Final report, primary data of the experiments and summary of calculations, University of Applied Sciences Wädenswil, Switzerland, unpublished
41. Voisard D, Meuly F, Ruffieux PA, Baer G, Kadouri A (2003) Biotechnol Bioeng 82:751
42. Christi Y (2001) Crit Rev Biotechnol 21:67

43. Davidson KM, Sushil S, Eggleton CD, Marten MR (2003) Biotechnol Prog 19:1480
44. Henzler HJ (2000) Particle stress in bioreactors. In: Schügerl K, Kretzmer G(eds.) Influence of stress on cell growth and product formation, series: advances in biochemical engineering/ biotechnology, vol.67. Springer, Berlin Heidelberg New York, p 38
45. Ho C, Henderson K, Rorrer G (1995) Biotechnol Prog 11:140
46. Kieran PM, Malone DM, MacLoughlin PF (2000) Effects of hydrodynamic and interfacial forces in plant cell suspension systems. In: Schügerl K, Kretzmer G(eds.) Influence of stress on cell growth and product formation, series: advances in biochemical engineering/biotech-nology, vol.67. Springer, Berlin Heidelberg New York, p 141
47. Leckie F, Scraggs H, Cliffe K (1991) Enzyme Microb Technol 13:801
48. Marks DM (2003) Cytotechnology 42:21
49. Nienow AW (1998) Hydrodynamics of stirred bioreactors. In: Pohorecki R (ed.) Fluid mechanics problems in biotechnology. App Mech Rev 51:3
50. Nienow AW (2006) Cytotechnology, DOI 10.1007/s10616-006-9005-8
51. Takeda T, Seki M, Furusaki S (1994) J Chem Eng Jpn 27:466
52. Kunas KT, Keating J (2005) Stirred tank-single-use bioreactor: comparison to traditional stirred tank bioreactor. bioLOGIC Europe May 2005. Geneva, Switzerland
53. Knevelman C, Hearle DC, Osman JJ, Khan M, Dean M, Smith M, Aiyedebinu Cheung K (2002) Characterization and operation of a disposable bioreactor as a replacement for conven-tional steam-in-place inoculum bioreactors for mammalian cell culture processes. 224th National Meeting of the American Chemical Society. American Chemical Society, Washington DC, USA (poster), Boston, MA
54. Heidemann R, Riese U, Lütkemeyer D, Büntemeyer H, Lehmann J (1994) Cytotechnology 14:1
55. Eibl D, Eibl R, Frefel J, Hans D, Jenny D (1996) Erprobung und Bewertung eines Zellkulturreaktors für die Produktion von Sekundärmetaboliten mittels pflanzlicher Zellen. Final report, Ingenieurschule Wädenswil, Switzerland, unpublished
56. Studer A (2003) Bioverfahrenstechnische Untersuchungen an einem 2 L-BIOSTAT® B Plus Zellkulturreaktorsystem der Firma Sartorius BBI Systems. Semester thesis, University of Applied Sciences Wädenswil, Switzerland, unpublished
57. Czermak P, Weber C, Nehring D (2005) A ceramic microsparging aeration system for cell culture reactors. Scientific report, FH Giessen, Germany, http://kmubser.tg.fh-giessen.de/pm/ IBPT/Czermak-et-al-Sparger.pdf. Cited February 21, 2008
58. Ries C (2008) Untersuchungen zum Einsatz von Einwegbioreaktoren für die auf animalen Zellen basierte Produktion von internen und externen Proteinen. Diploma thesis, Zurich University of Applied Sciences, Department for Life Sciences and Facility Management. Wädenswil, Switzerland, unpublished
59. Ries C (2008) Verfahrenstechnische Charakterisierung des Single Use Bioreactor 50 L von Thermo Fisher Scientific. Scientific report, Zurich University of Applied Sciences, Department for Life Sciences and Facility Management. Wädenswil, Switzerland, unpublished
60. Applikon biotechnology (2005) Application note: kLa values
61. Pechmann G (2002) Disposable Wirkstoffproduktion im Wave-Reaktor mit animalen Suspensionszellen. Diploma thesis, Hochschule Anhalt (FH), Germany, unpublished
62. Eibl R, Eibl D (2008) Application of disposable bag-bioreactors in tissue engineering and for the production of therapeutic agents. In: Kasper G, Pörtner R, van Griensven M (eds.) Bioreactor systems for tissue engineering, series: advances in biochemical engineering/bio-technology, vol. 110. Springer, Berlin Heidelberg New York (in press)
63. Bauer I, Lamp J, Eibl R (2007) Influence of BioWave's culture bag pre-treatment on CHO cell growth and protein expression in chemically defined minimal medium. Biotech May 2007. Wädenswil, Switzerland, (poster)
64. Eibl R, Bauer I (2007) Application note: cultivation of serum-free growing CHO XM 111 suspension cells in the BioWave 20 SPS or BIOSTAT CultiBag RM 20 (fed batch/feeding mode) operating with CultiBag RM 2 L
65. Eibl R, Rutschmann K, Lisica L, Eibl D (2003) BioWorld 5:22

66. Eibl R, Bauer I (2007) Application note: cultivation and SEAP secretion of serum-free grow-ing CHO XM 111 suspension cells in the BioWave 20 SPS or BIOSTAT CultiBag RM 20 (fed batch) operating with CultiBag RM 20 L
67. Mazur X, Fussenegger M, Renner WA, Bailey JE (1998) Biotechnol Prog 14:705
68. Eibl D, Eibl R (2002) Entwicklungsstand und trends in der Zellkulturtechnologie. In: Beckmann D, Meister M, Heiden S, Erb R (eds.) Technische Systeme für die Biotechnologie und Umwelt-Biosensorik. Erich Schmidt Verlag, Berlin, p. 255
69. Kaufmann H, Mazur X, Fussenegger M, Bailey JE (1999) Biotechnol Bioeng 63:573
70. Rhiel M, Eibl R (2004) Der Wave als System zur Prozessentwicklung für Proteinexpressionen. Biotech May 2004, Wädenswil, Switzerland
71. Wernli U, Eibl R, Eibl D (2008) Quest 5:18
72. Weber W, Fussenegger M (2005) Baculovirus-based production of biopharmaceuticals using insect cell cultures. In: Knäblein J (ed.) Modern biopharmaceuticals. Wiley VCH, Weinheim, p. 1045
73. Dietzsch C, Genzel Y, Reichl U (2007) Vero or MDCK cells for influenza A virus production in microcarrier systems? European BioPerspectives May 2007. Cologne, Germany (poster)
74. Kreis W, Baron D, Stoll G (2001) Biotechnologie der Arzneistoffe. Deutscher Apotheker Verlag, Stuttgart
75. Hibino K, Ushiyama K (1999) Commercial production of ginseng by plant tissue culture technology. In: Fu TJ, Curtis WR (eds.) Plant cell and tissue culture for the production of food ingredients. Kluwer Academic, New York, p. 215
76. Wink M, Alfermann AW, Franke R, Wetterauer B, Distl M, Windhoevel J, Krohn O, Fuss E, Garden H, Mohagheghzadeh A, Wildi E, Ripplinger P (2005) Plant Gene Res 3:90
77. Evans J (2006) Plant-derived drug. http://www.rsc.org/chemistryworld/News/2006/February/07020602.asp. Accessed 10 April 2007
78. Hess D (1992) Biotechnologie der Pflanzen. Eugen Ulmer, Stuttgart
79. Deus-Neumann B, Zenk HM (1984) Planta Med 50:427
80. Oksman-Caldentey KM, Hiltunen R (1996) Field Crops Res 45:57
81. Sharp JM, Doran PM (2001) Biotechnol Prog 17:979
82. Doran PM (2002) Properties and application of hairy root cultures. In: Oksman-Caldentey KM, Barz WH (eds.) Plant biotechnology and transgenic plants. Marcel Dekker, New York, p. 143
83. Curtis WR, Emery A (1993) Biotechnol Bioeng 42:520
84. Su W (2006) Bioreactor engineering for recombinant protein production using plant cell sus-pension culture. In: Dutta Gupta S, Ibaraki Y (eds.) Plant tissue culture engineering, series: focus on biotechnology, vol 6. Springer, Dordrecht, p. 135
85. Schwarz S (2004) Comparison of different scale-up methods for the production of high value-compounds in selected plant systems. Diploma thesis, University of Applied Sciences Wädenswil, Switzerland, unpublished
86. Zhong JJ (2001) Biochemical engineering of the production of plant-specific secondary metabolites. In: Scheper T (ed.) Plant cells, series: advances in biochemical engineering/bio-technology, vol. 72. Springer, Berlin Heidelberg New York, p. 1
87. Schürch C, Blum P, Zülli F (2007) Phytochem Rev, DOI 10.1007/s11101-007-9082-0
88. Griehl C, Isdepsky A, Krause-Hielscher S (2007) Establishment of marine macrophytic cell cultures for the production of bioactive metabolites. http://www.wavebiotech.net. Accessed 02 March 2008
89. Jouhikainen K, Lindgren L, Jokelainen T, Hiltunen R, Teeri TM, Oksman-Caldentey KM (1999) Planta Med 208:545
90. Cuperus S, Eibl R, Rischer H, Oksman-Caldentey KM, Cusidó RM, Pinyol MT, Eibl D (2007) Disposable bag bioreactor for plant cell and tissue cultures. PSE congress "Plants for human health in the post-genome era" August 2007. Espoo, Finland (poster)
91. Jablonski-Lorin C, Mellio V, Hungerbühler E (2003) Chimia 57:574
92. Hess S (2001) Kultivierung von *Erynia neoaphidis* im Wave-Reaktor. Scientific report, University of Applied Sciences Wädenswil, Switzerland, unpublished

93. Canales R, Hlubina M, Baier U, Tuor U (2001) Evaluation of cultivation parameters for mass production of *Erynia neoaphidis*. IOBC Meeting "Entomopathogens and insect parasite nematodes" June 2001. Athens, Greece (poster)
94. Hess S, Baier U, Lettenbauer C, Hafner D (2002) A new application for the Wave Bioreactor 20: cultivation of *Erynia neoaphidis*, a mycel producing fungus. IOBC meeting "Insect pathogens and insect parasitic nematodes" May 2002. Birmingham, U.K.
95. Hirschy O, Schmid T (1999) Flüssigkultivierung von entomopathogenen Nematoden. Scientific report, University of Applied Sciences Wädenswil, Switzerland, unpublished
96. Hirschy O, Schmid T, Grunder JM, Andermatt M, Bollhalder F, Sievers M (2001) Wave reactor and the liquid culture of the entomopathogenic nematode *Steinerma feltiae*. In: Griffin CT, Burnell AM, Downes MJ, Mulder R (eds.) Developments in entomopathogenic nematode/bacterial research. DG XII, COST 819, Brussels, Luxembourg
97. Hardy J, Priester P (2004) BioProc Int (Supplement series "The disposables option"):32

Disposable Bioreactors for Plant Micropropagation and Mass Plant Cell Culture

Jean-Paul Ducos, Bénédicte Terrier, and Didier Courtois

Abstract Different types of bioreactors are used at Nestlé R&D Centre – Tours for mass propagation of selected plant varieties by somatic embryogenesis and for large scale culture of plants cells to produce metabolites or recombinant proteins. Recent studies have been directed to cut down the production costs of these two processes by developing disposable cell culture systems. Vegetative propagation of elite plant varieties is achieved through somatic embryogenesis in liquid medium. A pilot scale process has recently been set up for the industrial propagation of *Coffea canephora* (Robusta coffee). The current production capacity is 3.0 million embryos per year. The pre-germination of the embryos was previously conducted by temporary immersion in liquid medium in 10-L glass bioreactors. An improved process has been developed using a 10-L disposable bioreactor consisting of a bag containing a rigid plastic box ('Box-in-Bag' bioreactor), insuring, amongst other advantages, a higher light transmittance to the biomass due to its horizontal design. For large scale cell culture, two novel flexible plastic-based disposable bioreactors have been developed from 10 to 100 L working volumes, validated with several plant species ('Wave and Undertow' and 'Slug Bubble' bioreactors). The advantages and the limits of these new types of bioreactor are discussed, based mainly on our own experience on coffee somatic embryogenesis and mass cell culture of soya and tobacco.

Keywords Box-in-bag, Coffee, Scaling-up, Slug bubble, Somatic embryogenesis, Somatic seedling, Temporary immersion, Wave and undertow

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Contents

Abbreviations

FW   Fresh weight
SB   Slug bubble
TIB   Temporary immersion bioreactor
WU   Wave and undertow

1 Introduction

For more than 20 years, Nestlé R&D Centre – Tours has been involved in in vitro cultivation of plant material, either for the production of metabolites by cell cultures [1–8] or for mass propagation by somatic embryogenesis [9–19]. Despite biochemical and physiological studies allowing improved experimental protocols at laboratory level, these improvements have seldom been matched by large scale or mass propagation of these plant materials. The gap between lab and production scales arises from the type and cost of the facilities necessary for scaling-up.

Plant micropropagation refers to the production of true-to-type plants from organogenic cultures (stem cuttings, axillary buds, meristem clusters, protocorm-like bodies) under aseptic and controlled environment. Somatic embryogenesis, which is the process for the development of embryos from somatic cells, offers a tremendous potential for mass propagation [20]. Current commercial applications are still restricted to a limited number of plant species because these methods are based on manual aseptic division of the plant tissues and their transfer on numerous small containers containing semi-solid media. In the 1985s to 1990s, studies described micropropagation scaling-up into two types of bioreactor: those in which the cultures are continuously submerged and those in which the cultures are temporarily immersed in the medium (temporary immersion bioreactor). The first approach consists in growing plants tissues in fermentation vessels either mechanically agitated (stirred tank bioreactor, rotating drum bioreactor) or pneumatically agitated (bubble column bioreactor, air-lift bioreactor) [21, 22]. The second involves placement of plant

tissues on solid supports which are periodically perfused with nutrients solutions [23–26]. Reports on micropropagation using disposable bioreactors remain rare as confirmed by two books reviewing the recent advances in plant tissue culture engineering in liquid cultures [27, 28]. Mainly based on our own experience on coffee somatic embryogenesis, the present chapter will describe the status of this technique and discuss its advantages.

The culture of undifferentiated plant cells for the production of metabolites has been achieved at an industrial scale by various groups since the late 1970s, in conventional stainless stirred bioreactors, up to 75 m^3. In spite of the interest in this technology, and numerous scientific and technical advances, there are very few examples of economical production of metabolites [29, 30], such as the red dye shikonin [31], ginseng cells [32], berberine [33] and more recently taxus-derived drugs [34].

Recently, interest in mass plant cell cultures increased again, using plant cell as a host cell for the production of recombinant proteins [35–40] and as an alternative to genetically modified plants in field ('biopharming'). In January 2006, the USDA Centre for veterinary biologics approved Dow AgroSciences vaccine (virus in poultry) produced in tobacco plant cell cultures in bioreactor. Also in 2007, and for the first time, the FDA gave approval to Protalix (Israel) to begin clinical trials for a drug produced in carrot cell cultures [41], which demonstrates a renewed interest in plant cell culture systems for the production of biopharmaceuticals. Also food flavourings have been recently produced by hairy-root of *Catharanthus roseus* cells [42], as well as indigo precursor indican in genetically modified tobacco plants and cells cultures [43] and therefore considered as an alternative to genetically modified plants in field.

Nevertheless, the limited development of the technology is mainly due to plant cell low growth rate (doubling time currently between 24 and 48 h) and often low productivity: one cannot expect more than 10–15 batches per year per bioreactor and, even with continuous systems, the cost of the produced biomass remains high, limiting this use to very high value products. Usual equipment and support facilities associated with aseptic bioprocess are extremely expensive, partly because large-scale production is based on stainless steel vessels, sterilized in situ. For plant cells, some estimation has been made concluding that more than 60% of the production costs are due to the fixed costs: high capital costs of fermentation equipment, depreciation, interest and capital expenditure [44]. Running costs are also high due to low yields and the need to clean and sterilize the bioreactor after each culturing cycle.

Economical viable solutions for large scale vegetative propagation of various plant species or in vitro mass cell cultures demand improved selection of the highest producing strains or the high propagation ability, determination of the right physiological conditions for growth and production, and cheaper facilities for the cultivation itself.

This is why we have recently developed new, cheap, disposable equipment for the cultivation of undifferentiated plant cells of various species and the vegetative propagation via somatic embryogenesis.

2 Micropropagation and Somatic Embryo Cultures

2.1 Bioreactors for Plant Propagation

2.1.1 Usual Bioreactors

Production of about 10,000 shoots per batch in 10- to 20-L stainless steel or glass bioreactor has been reported [21, 22]. This type of culture has often been criticized because of the limitation of oxygen exchange, problems of excessive foaming and high shear stress. Moreover, a well known and major issue is anomalous morphogenesis when green propagules are grown in liquid media, due to hyper-hydricity of leaves and stems, greatly affecting the plant survival after transplanting. Particularly, for dicotyledonous plant species, most shoots are etiolated, succulent and easily damaged by handling and environmental stress when they are transplanted to the soil [22]. The submerged type bioreactor is usually used for high-density multiplication of cultures where submersion does not result in abnormal plant development, such as the proliferation of storage organs (bulbs, corms, microtubers), meristematic clusters, embryogenic callus and small size somatic embryos [45].

In the past, we have used stirred-tank bioreactors to investigate critical parameters for the success of torpedo stage embryo production of carrot and coffee somatic embryos [11, 12]. For both species, we observed that constant submergence in liquid media completely inhibited leaf development from somatic embryos, even if the biomass was diluted. To extend the development of the embryos beyond the torpedo stage, it was necessary to subculture them onto gelose medium for their development into plantlets suitable for acclimatization. Another drawback of these bioreactors is their low performance regarding light transmittance through the biomass. When the purpose is to produce micro-plants ready for transplantation in the soil, high illumination is required in the bioreactor. However, among the different technologies involved in bioreactor engineering for plant propagation, the most difficult is the introduction of light into the biomass [22, 45].

2.1.2 Temporary Immersion Bioreactor

To avoid the problems associated with submerged cultures, a new type of bioreactor appeared in the late 1980s. These pieces of equipment were constructed to allow cycling of the culture medium, thus exposing the plant tissues to the liquid media intermittently rather than continuously. They have been used for shoot cultures of cow tree [23], *Pinus radiata* [24] and serviceberry [25, 26]. These temporary immersion bioreactors (TIB), also termed temporary immersion systems (TIS) or 'Ebb and Flow methods', offer the advantages of cultures in liquid medium, therefore reducing labour cost without the disadvantages of a liquid environment.

Thereafter, different versions of TIB have been developed: nutrient mist bioreactors [46], tilting and rocking vessels [47, 48] or single containers with two compartments, the

upper one containing the tissues, such as the so-called Recipient for Automated Temporary Immersion (RITA®, Vitropic, France) [49]. This small bioreactor has been used to produce torpedo-shaped embryos of *Coffea arabica* F1 hybrids [50, 51], tea [52] and sugarcane [53]. However, a system consisting of a pair of bottles connected by a silicone tube, known as the twin flask system [54], is generally preferred because construction and operation are very simple. Easy to scale up to 10–20 L, it represents a very attractive low-cost alternative. This typical design consisting of two vessels (plastic or glass), one holding the liquid medium and the other the cultures, becomes more and more popular for large-scale propagation. Air pressure is applied to push the medium from one container to the other to immerse the explants or to withdraw the medium. This process is repeated at preset intervals, and can be easily automated. It has been used for shoot multiplication of pineapple and various other tropical crops [54, 55], *Phalaenopsis species* [56, 57], and *Prunus* and *Malus species* [58]. These simple pieces of apparatus were also used for the optimization of secondary metabolite production from shoots of diverse species, such as *Ruta graveolens* or *Hypericum* [59, 60].

In our laboratory, we have implemented the Temporary Immersion Bioreactor for the scaling-up of coffee embryo conversion from torpedo to cotyledonary stages. This step, pre-germination, is mainly characterized by the greening and the acquisition of photo-autotrorophic characters. Our TIB version is similar to the twin flask systems. It consists of two glass jars (Fig. 1a) [17, 18]: a 10-L jar con-
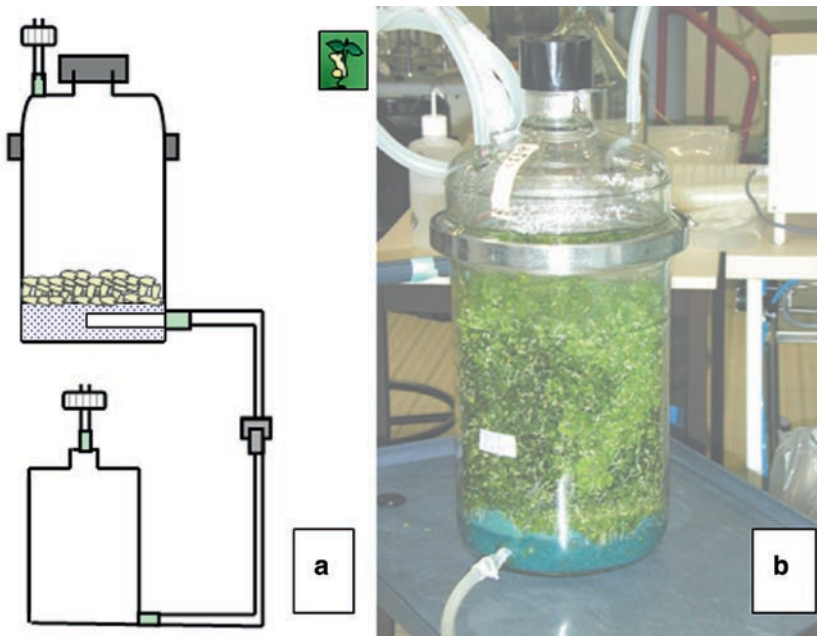

Fig. 1a,b Glass jar temporary immersion bioreactor. **a** Diagram. **b** View of a 10-L bioreactor at the end of the pregermination phase

taining the somatic embryos (20 cm diameter × 30 cm height) and a 5-L bottle containing the medium and placed below the 10-L jar and connected to an air pump. When the pump is turned on, the pressure pushes the liquid medium to the upper part. When the pump is switched off, the medium flows back down due to gravity. The main characteristic of this TIB is a polyurethane foam disk laid on the bottom of the 10-L jar. Between the immersion periods this disk isolates the embryos from the thin liquid medium layer which remains in the vessel. It retains about 1 L of liquid medium inside the vessel, and therefore maintains a sufficient relative humidity (85–90%). During the immersion, this disk has the function of an air sparger and facilitates the good ventilation of the headspace.

Temporary immersion culture brings several advantages [61]. It insures adequate oxygen transfer because the tissues are not permanently immersed in liquid media in which oxygen is poorly soluble. Shear stresses are almost suppressed due to the lack of mechanical agitation or permanent aeration. The hyper-hydricity is limited and can be controlled by manipulating the frequency and duration of immersions.

Nevertheless, targeting commercial production, the current TIB systems have to be improved, due to some limitations, such as the size of the vessels and their disposability. We encountered issues with insufficient mixing which led to the accumulation of coffee embryos forming compact aggregates. Furthermore, for some Robusta clones, the top of the biomass reaches the cover of the vessel (Fig. 1b). In this case, about 20,000 transplantable embryos can be collected from such cultures. The embryos present a large heterogeneity in size, from precocious (1 mm) to the fully expanded cotyledon stage (20 mm). Approximately, only half of the embryos have a hypocotyl larger than 5 mm which is the main criterion to select the embryo at the sowing time in the greenhouse. Most likely a non-uniform light distribution inside the TIB may be responsible for differences in growth and quality among embryos. When shoots are grown at a high density in a bioreactor and are illuminated externally, light becomes a rate-limiting factor as it can only penetrate a few centimetres through the compact biomass [45].

Consequently, we looked after large polycarbonate containers offering a greater surface-to-volume ratio to overcome light limitation. Unfortunately, these trials were not successful due to frequent contaminations at the level of the cover ring and also because of the deformation of the polycarbonate with repeated autoclaving. Therefore, the use of glass or rigid plastic TIBs at a commercial scale is possible but with limited size and performances.

2.1.3 Disposable Bioreactors

Disposable containers, up to 0.5-L, are commonly used for industrial micropropagation on semi-solid media. They are made of rigid polyethylene and bulk sterilized by gamma radiation but they are not adapted for culture in liquid medium.

In India, a commercial facility was adapted for the production of shoots of sugarcane to a new system which uses 20× 30 cm polypropylene bags, without any outlet and inlet ports [62]. After pouring 30 mL of sterile liquid medium under the

laminar flow, the bags are inoculated with single plants which grow into a bunch of plants within 1 month. The bags are closed with a heat-sealing machine and hung on ropes in a greenhouse under natural light illumination. In a 50 m² greenhouse, 100,000 bags can be accommodated. According to the authors, once closed, the polypropylene bags contain enough oxygen for the cultures. To avoid oxygen limitation in non-ventilated bags, disposable vessels have been developed using fluorocarbon polymer films (Neoflon® films, Daikin Industries, Ltd) [63]. This device, referred to as the 'Culture Pack', consists of a 3-L box-shaped culture vessel with an external stainless steel frame. Due to the fluorocarbon polymer gas permeability, the culture pack system has no adverse effect on shoot development of *Cymbidium* and *Spathiphyllum.* Each vessel can contain 16 shoots growing on agar medium or on rock wool plugs soaked with liquid medium.

The first disposable apparatus suitable for bulk-cultivation of propagules cited in the literature is an airlift bioreactor: it is made of non-autoclavable clear flexible plastic film, sterilized by gamma radiation, and has a vertical and conical shape [64, 65]. This so-called LifeReactor® (Osmotek LTD, Rehovot, Israel) has a working volume of 1 or 5 L and contains a sparger for bubble production. Meristematic clusters were cultured in this disposable bioreactor to grow propagules of potato, fern, banana and gladiolus [64]. The clusters' biomass increased five- to eightfold within 1 month. At the end of the culture, the propagules must be subcultured onto agar medium for their development into plants which can be transplanted to the greenhouse. This bioreactor has been adapted for temporary immersion culture just by coupling two unit devices (Ebb and Flow Bioreactor®, Osmotek LTD, Rehovot, Israel). For laboratories with limited resources, as in developing countries, a procedure to make a simple version of 1 L or less of this temporary immersion bioreactor was described [66].

Recently, commercial implementation based on 5- to 10-L autoclavable plastic bags was mentioned for two major propagation laboratories in North America but no details were given [67]. To produce metabolites, embryogenic calli of *Allium sativum* were grown in 2-L plastic bags by wave-induced agitation [68].

As a conclusion, reports on micropropagation using disposable bioreactors made of flexible plastic still remain much less frequent than those describing polycarbonate devices, such as RITA® or twin flask systems.

2.2   Box-In-Bag Bioreactors

By providing a larger surface-to-volume ratio, a horizontal design is more convenient than a vertical one to produce micro-plants that can be directly transplanted from the bioreactor to the greenhouse. However there is a serious concern about how to maintain a headspace between the immersion periods in a large and horizontal TIB made of flexible plastic. This point can be solved by developing three types of containers: (1) 3D structures such as cubes, (2) 2D bags with an external frame, and (3) 2D bags with an internal frame. Cubic structures were tested but their fabrication was found
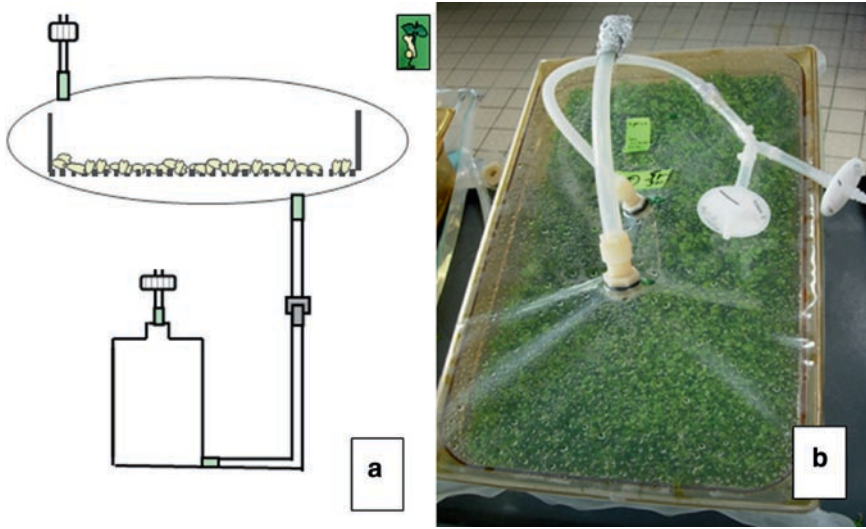
Fig. 2a,b Box-in-bag temporary immersion bioreactor. **a** Diagram. **b** View of a 10-L bioreactor at the end of the pregermination phase

to be too expensive for micropropagation purposes. In 2006, at the 27th International Horticultural Congress (Seoul), we presented a very simple solution corresponding to the third option and consisted of placing a rigid box inside a plastic bag (Fig. 2) [16]. This so-called 'box-in-bag' bioreactor is easier to handle than an empty bag having an external frame. We describe a detailed account of how to prepare an example of this bioreactor for the pre-germination of coffee somatic embryos.

Bags are made from a transparent plastic film composed of polyethylene and nylon (CPL613, Charter Medical, Lydall Group, NC, USA) and are supplied closed on three sides. They are 750× 420 mm in size and have two polyethylene ports moulded into the film. The port A (12 mm diameter) is positioned above the bag; it is used for the inoculation step and then for air outlet (Fig. 3a). The port B (7 mm diameter) is located below the device and used for air inlet and medium entrance and exit.

A 50 × 30× 10 cm rigid box made of a transparent and ionisable plastic, for instance polycarbonate (Gastronorm 1/2, Cambro, Huntington Beach, USA), is introduced without its cover into the bag. The bottom of the box is perforated with 1–1.5 mm diameter holes, or better, with two 50 mm diameter holes in which 90 × 30 mm polyurethane foam disks are fixed (Tramopen 45 ppI, Javaux, Maintenon, France) (Fig. 3b). One of the foam disks is located just above port B, which functions as both the medium inlet and air entrance inlet. A funnel made of silicone tubing is fixed through this disk to permit the rapid introduction of the medium inside the box at the beginning of the immersion periods. Silicone tubing is fixed to each port and female polycarbonate connectors and plugs (Cole Parmer, Minneapolis, USA) are placed at their extremities (Fig. 3c). The fourth side is heat sealed and the system gamma-sterilized (Ionisos, Sablé, France).
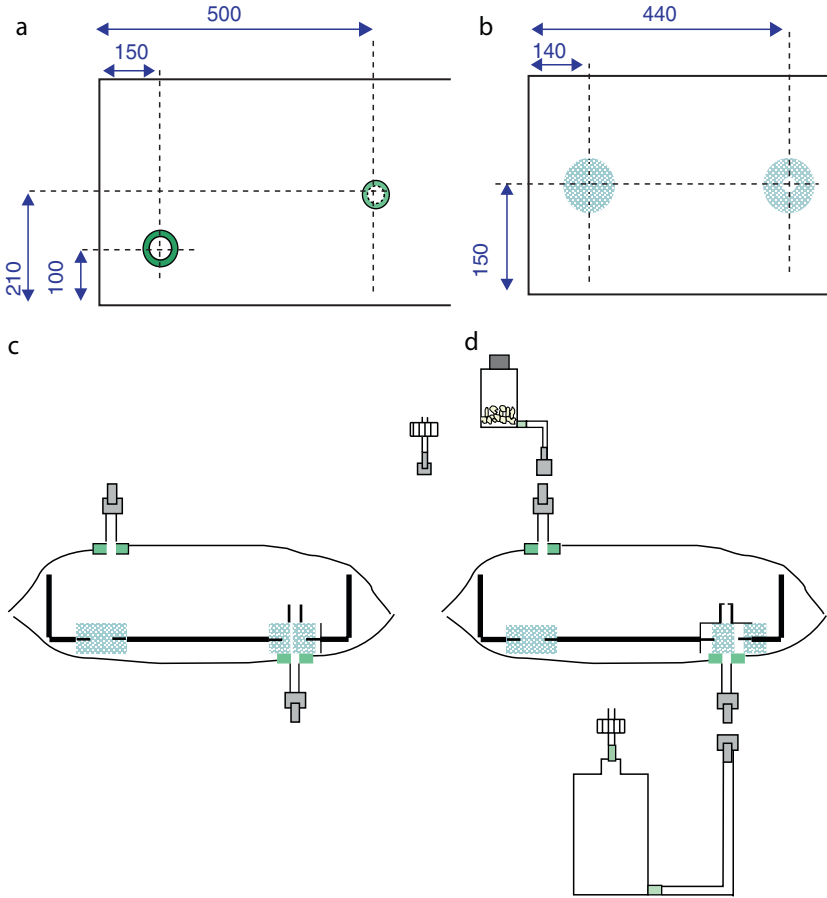
Fig. 3a–d Details for the preparation of a 10-L box-in-bag temporary immersion bioreactor. **a** Bag. **b** Box. **c** Closing the bioreactor and preparation for sterilization. **d** Inoculation and connection to the medium vessel

The torpedo-shaped embryos are aseptically introduced into the bioreactor by using an inoculator bottle connected to port A (Fig. 3d). A 0.2-µm sterilizing air filter (Midisart or Sartofluor, Sartorius, Germany) is then connected to this port. The glass bottle containing 5 L of autoclaved medium is connected to port B. The whole system is placed in the culture room, the medium tank beneath. The later is connected to a compressed air source. During the culture, overpressure at 0.5 bar is applied through the 0.2-mm vent filter of the reservoir bottle at repeated intervals, generally twice a day for 6 min, forcing the medium into the bag. In these conditions, 5 L of medium can be transferred in 1 min from the reservoir to the bioreactor. Fresh air is then injected inside the bag which is inflated over a period of 5 min. Using CO_2 as a gas tracer, it has been checked that this period of time is sufficient to refresh fully the atmosphere.

2.3   Case Study: Coffee Somatic Embryogenesis

During the 1990s, three major progress steps led to the scaling up of somatic embryogenesis of the allogamous species *Coffea canephora* (var. Robusta) by reducing the labour cost input: (1) culture of embryogenic cells and torpedo stage embryos in liquid media [9, 10, 12], (2) pre-germination from the torpedo to the cotyledonary stage by temporary immersion in liquid media [50], and (3) ex vitro germination by directly sowing cotyledonary stage embryos, without true leaves, in the greenhouse [14, 69]. From 1996 to 2000, large-scale Robusta field trials were set up representing a total of 12,000 somatic seedlings from ten clones. The trees did not show major undesired somaclonal variation and no significant differences were seen between the somatic seedlings and the microcutting-derived trees for the observed morphological traits and the yield characteristics [15, 17].

Based on the progress mentioned above, a pilot process for large-scale production of pre-germinated Robusta somatic embryos was implemented in our centre [18]. Three operators can produce a total of 3.0 million pre-germinated embryos per year using 10-L glass TIBs. A production cycle is started every month and requires a total of 4–5 months to produce somatic embryos ready to be sown in the greenhouse. A cycle can be summarized as follows:

–   Each run starts from 60 g FW of embryogenic cells multiplied in liquid medium.
–   For the production of torpedo stage embryos (Fig. 4a), the cells are transferred into submerged cultures, stirred-tank bioreactor or Erlenmeyer flasks, inoculated at a density of 1.0 g L−1.
–   For pre-germination up to cotyledonary stage (Fig. 4b), the torpedo stage embryos are transferred into 35–40 10-L glass TIBs. Each bioreactor is inoculated with 30–60 FW g of embryos. When most of the embryos turn green, generally within 2–4 weeks, the medium is replenished by fresh medium.
–   Within 2–3 months, a total biomass of about 7–9 kg FW is collected from the bioreactors, corresponding to 2–250,000 cotyledonary embryos.

A significant part of the labour is devoted to the handling and the cleaning of the glass jars. However, this material is heavy and breakable. In 2005, we started trials to develop bioreactors made of flexible plastic film. By a step-by-step approach, these trials led us to the box-in-bag design (Fig. 4c). At the end of the pregermination phase, the box-in-bag bioreactor can be easily cut to harvest the embryos (Fig. 4e). We compared its performance to the 10-L glass jar TIB by inoculating the bioreactors with the same quantity of embryos issued from the same cell lines. The biomass and the number of embryos having a hypocotyl longer than 5 mm are significantly higher than in the 10-L glass vessel (Table 1), probably because the area is 1,260 cm², i.e., four times the area of the 10-L glass jars for a similar volume. The embryos look very green and their ability to develop a plant is similar to those grown in the glass bottles. This experiment confirms the importance of light intensity on embryo quality, as they are greener and taller under higher light intensity conditions. In 2008, we are planning to replace all the glass vessels by such disposable containers. A significant increase in term of embryos produced per operator, about 50%, is expected.
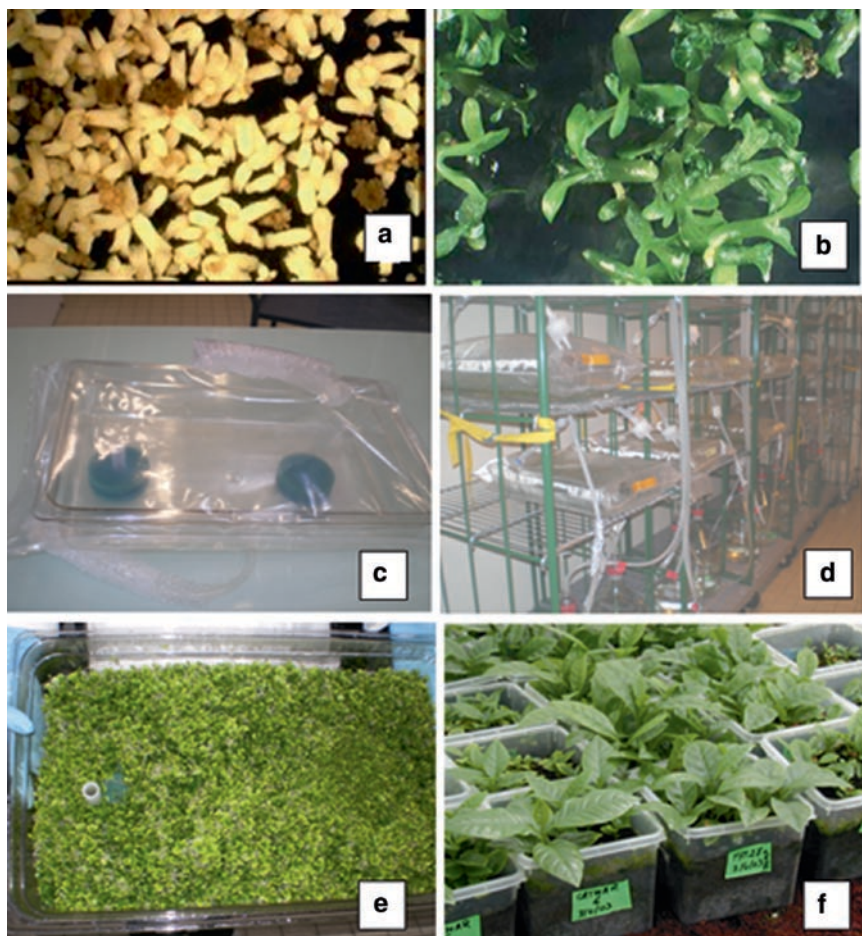
Fig. 4a–f Pilot process for the production of pre-germinated Robusta embryos. **a** Torpedo-stage embryos. **b** Cotyledonary-stage embryos. **c** A 10-L box-in-bag disposable bioreactor before inoculation. **d** Overview of a culture room with disposable bioreactors. **e** View of cotyledonary embryos produced in a disposable bioreactor. **f** Ex vitro germination for the conversion to fully developed plantlets

Table 1 Comparison of two types of 10-L temporary immersion bioreactors for the pre-germination of Robusta somatic embryos (clone FRT23) (reproduced from [16])

TIB type	Pregermination		Ex vitro germination
	FW[a]	Pregerminated embryos[a]	Embryo-to-plantlet conversion rate[b]
	g/TIB	nbr/TIB	%
Glass jars	519	18,576	42
Box-in-bag	943	26,794	57
	S[c]	S[c]	NS

[a]Means of three experiments
[b]Means of five replicates of 25 embryos
[c]S: significant ($P < 0.01$)

Considering germination tests conducted under a plastic tunnel in our greenhouse (Fig. 4f), our current production capacity is enough to regenerate potentially about 1.0 million plantlets. This potential can be increased up to 1.5–2.0 million if the ex vitro germination is conducted under a microenvironment, achieved by placing a transparent cover 2–3 cm above the embryos which would benefit from the positive effect of the CO_2 released by horticultural media as peat or coconut fibres [19]. The embryos are sent to coffee producing countries where they are sown in ex vitro conditions under a tunnel plastic to develop plantlets bearing true pairs of leaves within 4–6 months. The plantlets are then grown in polyethylene bags in the nurseries during 6–12 months before their transplantation to the field [18, 70].

2.4   Advantages and Limitations

The box-in-bag disposable TIB combines the advantages provided by the two types of plastics, rigid and flexible. The rigid plastic box facilitates the manipulations, maintains a culture headspace between the immersion periods and allows a horizontal distribution of the biomass, allowing better oxygenation and illumination. Moreover, the possibility of stacking several boxes one top of another makes this system easy for transportation: it is possible to send in vitro plants keeping them inside the bioreactor in which they have grown. The international exchanges of sterile plant material are therefore greatly facilitated. The flexible plastic is a disposable device (low cost, simple to operate) and offers a high process security and a great versatility by allowing a large diversity in sizes and designs. Both plastics can be used together as a mini-greenhouse for storage, shipment, hardening, and probably even for ex vitro germination under microenvironment conditions.

The box-in-bag TIB is very easily scalable because its size can be increased without the cost impact of custom-made moulders; a lot of rigid and translucent plastic boxes of different sizes are commercially available, for instance from gathering retailers. In order to illustrate the versatility it offers in design, we present a very simple TIB consisting of a bag containing both embryos and medium (Fig. 5). The immersion is simply achieved by manually moving the box into the medium.

The security of disposable devices allows the implementation of the cultures in greenhouses instead of expensive culture rooms. Moreover, growth under natural illumination is a relevant strategy to insure an excellent survival rates when the in vitro plants are transferred to the soil [62]. The box-in-bag system also offers the possibility for bulk-cultivation of coffee somatic embryos in photoautotrophic conditions, i.e., without sugar and with CO_2 enrichment. This culture method improves the quality of the vitroplants when they are grown individualized on gelose media or plugs [71–74]. Obviously, cultures conducted under photoautotrophic conditions will only be efficient if the embryos are enough illuminated.

Unsatisfactory mixing sometimes remains a sticking point in this large TIB. Nevertheless, if it is necessary to disperse the immersed embryos, the operator can easily move the bag when it is inflated due to its light weight. It is not totally
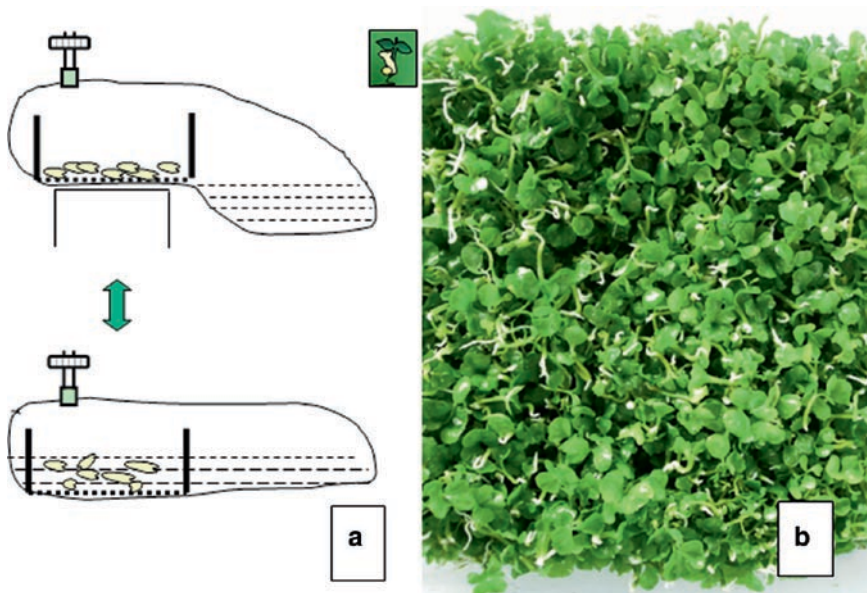
Fig. 5a,b Very simple temporary immersion bioreactor. **a** Diagram. **b** View at the end of the pregermination phase

disposable because some components are too expensive, as the connector systems. Moreover, it is not available 'ready-to-be-used' yet. We are investigating different sizes and designs of ready-to-be- used bioreactor manufactured by Hegewald Medizinprodukte (Lichtenberg, Germany).

3 Mass Plant Cell Culture and Metabolite Production

3.1 Disposable Bioreactors for Plant Cells

In order to minimise production costs, a few alternatives to traditional stainless steel bioreactors have been developed [75–77]. Singh [75] developed a disposable bioreactor with an original agitation apparatus, using an inflated bag placed on a rocking mechanism that induces a wave-like motion to the liquid contained therein. This system is mainly used for animal cell cultures. Few papers have been published in plant cell domain, and only with small working volumes [68, 78, 79].

We are developing two new flexible, scalable, plastic disposable bioreactors [8]. The first is based on the principle of a wave/undertow mechanism providing convenient mixing and aeration to the plant cell culture ('WU bioreactor'). The second is a new bubble column bioreactor that allows an easy increase of working volumes (up to several hundred liters) with the use of multiple units ('SB' bioreactor). Both

systems are pre-sterilized and have been designed to allow for medium introduction, inoculation and sampling.

To assess the performances of these two new systems, they will be compared in terms of biomass accumulation (tobacco and soya) and isoflavone production (soya) to two so-called 'traditional' systems: Erlenmeyer flasks and a 14-L stirred tank bioreactor, which are widely used in the laboratory.

3.2 Wave and Undertow and Slug Bubble Bioreactors

3.2.1 Description

WU

The Wave and Undertow (WU) bioreactor consists of a large flexible plastic container partly filled with medium and inflated with air (Fig. 6). The system is located on a horizontal table equipped on one side with a moveable platform. The intermittent rising movement of the platform to the rest point, and down/descending movement back to initial position enable continuous mixing and aeration through the wave/undertow
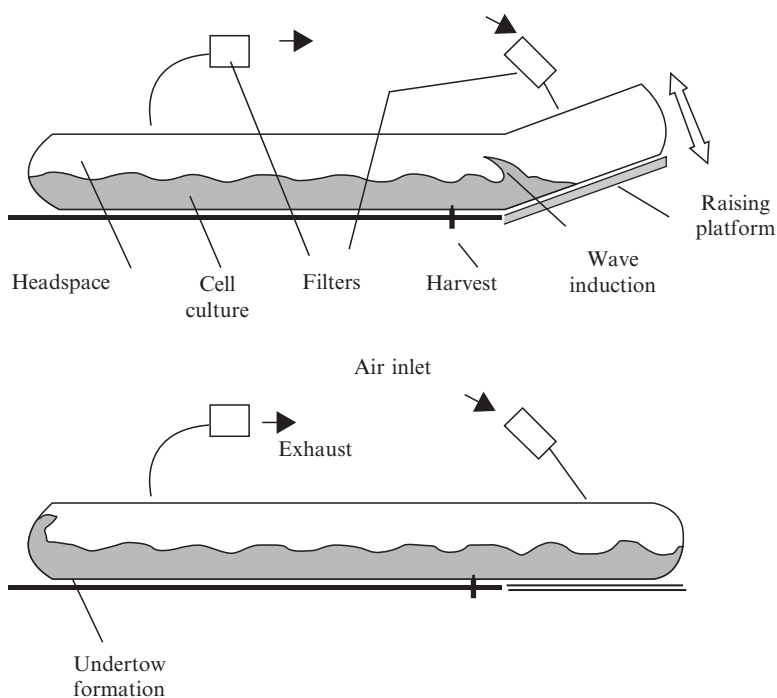
Fig. 6 Schematic diagram of the WU bioreactor (reproduced from [8])

motion. The platform ascension leads to the formation of a wave that propagates through the bag and bounces off the extremity creating an undertow which returns to the initial point. This action is repeated, creating a new impulse to ensure persistence of flow within the WU bioreactor. Sterile air is continuously fed in the headspace. Wave/undertow induction provides liquid culture mixing and bubble-free aeration. Oxygen transfer is accomplished by transport from the headspace air to the liquid culture.

SB

The Slug Bubble (SB) bioreactor consists of a vertical flexible plastic cylinder filled with medium up to circa 80% of its height (Fig. 7). Agitation and aeration are achieved through the intermittent generation of large cylindrical single bubbles at

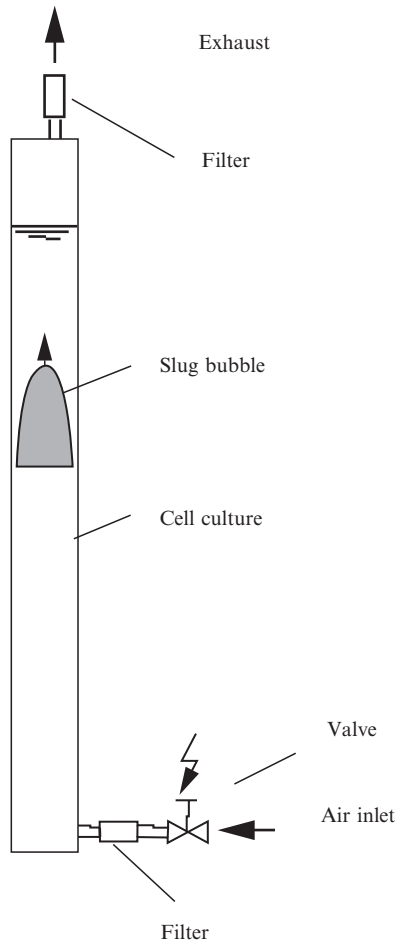**Fig. 7** Schematic diagram of the SB bioreactor (reproduced from [8])

the bottom of the system that rise to the top of the cylinder. These bubbles are comparable to 'Taylor bubbles', or 'slug bubbles' [80–82]. These bubbles can be described as long bullet-shaped bubbles, which nearly occupy the entire cross-section of a pipe. The rear of the slug bubble is a region characterized by strong mixing, where all transfer processes are enhanced. Mixing and oxygen transfer are therefore achieved at the same time.

3.2.2   Engineering Aspects (Manufacturing/Working)

Both systems are entirely made of plastic components. They are either 'home-made' or contract-manufactured and they are designed to allow air inlet, medium introduction, inoculation, sampling and air outlet. They are maintained in an air-conditioned room for temperature control.

WU

With regard to manufacturing, as the wave generation (which is the basis of the agitation) leads to the bioreactor to be regularly bent (always at the same spot, from 200,000 to more than 1 million times in a batch), the plastic film used to manufacture the WU needs to be flexible and capable of enduring this repeated movement. Two types of plastic films have been found that can satisfy that demand: PVC (polyvinylchloride) and PU (polyurethane).

Most systems have been built in-house from biopharmaceutical grade PVC (Achilles, WA, USA); however this film displayed two main disadvantages. First, it was so pliable that it loosened during system manufacture and during pressure test leading to a non- symmetrical and extensible systems (that is of variable volume for given lengths). Second, it was not flexible enough to avoid perforations, followed by leaks, at the weakest points. Two PU films (a polyurethane ester and a polyurethane ether, manufactured by Epurex Films, a Bayer MaterialScience company) have also been used. However, whilst being flexible, they do not show the extreme extensibility displayed by the Achilles PVC, which permits bags to be manufactured more easily and of constant volume. Finally, another PVC film, 'Transfufol', provided by the company Lider, to whom the making of WU bags was contracted, has also been used. For sterilisation, PVC films are autoclaved (Fedegari, Italy) for 40 min at 121 °C. PU films are sterilized by gamma radiation (12–25 kGy).

With regard to working, the pre-sterilized system is set up on the table and undergoes a pressure test (air filling) to check for potential leaks. If the bag is intact, medium can be added and the system prepared before inoculation. Platform movements are simply achieved by pneumatic jacks located under the platform. The time needed to allow for the platform to rise and stay up (T1), and the time necessary for the platform to descend and stay down (T2) can be adjusted easily. Other parameters are adjustable: the percentage of culture volume located on and lifted by the platform(s) (v), the platform raising angle (α), and the air inlet flow rate (Q). The agitation

Table 2 WU bioreactor volumes and dimensions

Total volume (L)	Length (L) (cm)	Width (W) (cm)	L/W	Working volume (L)	Filling level (%)
60	175	35	5	20	33
				30	50
200	280	55	5	70	33
				100	50
750	390	75	5	250	33

Table 3 SB bioreactor volumes and dimensions

Total volume (L)	Working volume (L)	Diameter (D) (cm)	Floor surface (cm²)	Height (cm)	Unaerated suspension height (cm)	Aspect ratio (H/D)
14	10	8.5	60	250	175	21
24	20	11.0	100	250	210	19
64	50	18.0	250	250	200	10
90	70	18.0	250	350	280	15
135	100	20.2	320	350	310	21
175	125–150	22.5	400	420	315–380	14–17

intensity is adjusted depending on the batch volume and on the oxygen demand of the cell strain (Table 2).

SB

With regard to manufacturing, the system is made from biopharmaceutical grade polyethylene (CPL613; Charter Medical, Lydall Group, NC, USA) and gamma-sterilized (Ionisos, France) or produced and pre-sterilized by Charter Medical using the same flexible plastic film.

With regard to working, the pre-sterilized system is set up in a rigid plastic tube and undergoes a pressure test (air filling) to check for leaks. As previously, if the bag is intact, medium can be added and the system prepared before inoculation. The slug flow regime is artificially produced by intermittent gas supply, using a solenoid valve and compressed air. The valve relieves a predetermined quantity of air at the given frequency. The quantity of air can be adjusted by changing the inlet pressure (P), the valve opening duration (T1), or the bubble frequency (f). The usual inlet pressure is from 0.03 to 0.05 MPa for 10–70 L (working volume) reactor. The corresponding averaged flow rates vary between 0.1 and 0.5 vvm, which is consistent with values usually encountered with plant cell culture [29]. The aeration intensity is defined according to the batch volume thanks to the programming device. The rigid plastic tube (PVC) maintains the reactor vertical. A horizontal slot (3–7 cm wide) is cut up lengthwise for the crossing of different inlets and outlets and the observation of the culture. Table 3 presents different sizes of SB bioreactors.

For both systems, aeration is achieved with compressed air sterilized trough membrane air filter (Sartofluor, Sartorius AG, Germany). Culture medium is either sterilized by autoclave and aseptically transferred to the systems, or sterilized by membrane filtration (Sartobran, Sartorius AG, Germany).

3.2.3 Characteristics ($k_L a/O_2$ Transfer)

The volumetric oxygen mass transfer coefficient ($k_L a$) of the Erlenmeyer flask, stirred-tank bioreactor, WU and SB systems were measured in duplicate by the dynamic gassing-out (air) method using a polarographic, temperature-compensated, dissolved oxygen probe. The liquid (water) in the system is deoxygenated by gassing nitrogen through the inlet filter. When the dissolved oxygen (DO) probe (Ingold) reached 0% saturation, aeration and agitation were started and the rising DO is recorded as a function of time. The value of $k_L a$ is defined as:

$$\frac{dC}{(C^*-C)} = k_L a.dt$$

where C: oxygen concentration in the liquid at time t (mg L^{-1})
C*: oxygen solubility in the liquid (mg L^{-1})

For the Erlenmeyer flasks, the same procedure was used, the only difference being that the O_2 probe is a Clark oxygen electrode immersed in the chamber body of an oxygen system, through which the liquid from the Erlenmeyer flask circulates along a closed circuit by a peristaltic pump. This system avoids the perturbations provoked by the direct immersion of the probe in the Erlenmeyer flask.

Oxygen mass transfer coefficients measured in the WU bioreactor (Table 4) were lower than those observed in a traditional stirred tank bioreactor, but comparable to or higher than those encountered in Erlenmeyer flasks or other known flexible disposable cell culture systems such as the Wave Bioreactor [68, 75].

Table 4 Volumetric mass transfer coefficient ($k_L a$)

Culture system	Working volume	Agitation/aeration				$k_L a$ (h^{-1})
250-mL Erlenmeyer flasks	100 mL	100 rpm				5
14-L stirred-tank bioreactor	10 L	0.04 vvm; 150 rpm				3
		0.25 vvm; 200 rpm				15
		0.5 vvm; 100 rpm				29
					vvm	
60-L WU	20 L	At given conditions			0.12	10
60-L WU	30 L	At given conditions			0.18	9
		P	T1	T2	vvm	
24-L SB	20 L	0.04	0.5	7.0	0.31	7
			0.5	4.0	0.49	16
64-L SB	50 L	0.05	0.3	7.0	0.17	10
			0.5	7.0	0.21	17

vvm: air flow rate (vvm); P: air inlet pressure (MPa); T1: valve opening duration (s); T2: time interval between two successive valve openings (s)

The Slug Bubble bioreactor showed good oxygen transfer capacities. Oxygen transfer coefficients (Table 4) were comparable to coefficients for traditional culture systems such as Erlenmeyer flasks and traditional bioreactors in the range of agitation and flow rates compatible with the oxygen demand and low shear stress required for plant cell cultures. As it might have been expected, these results also show that increasing the valve opening time (bubble size) or the opening frequency (bubble frequency), both leading to the increase of the average gas flow rate, resulted in higher $k_L a$ values.

3.3 Case Studies: Tobacco and Soya Cell Cultures

3.3.1 Plant Material, Methods

The tobacco cell strain and the isoflavone-producing soya strain are grown in 250-mL Erlenmeyer flasks containing 100 mL medium on a gyratory shaker (New Brunswick Scientific, USA) at 100 rpm (shaking diameter 20 mm), at 25 °C in darkness. The *Nicotiana tabacum L* BY2 cell strain [83] is grown in MS components [84] with KH_2PO_4 (270 mg L^{-1} instead of 170 mg L^{-1}), 0.2 mg L^{-1} of 2,4-dichlorophenoxyacetic acid and 30 g L^{-1} of sucrose, at pH 5.8 and subcultured every week. The *Glycine max* (L.) Merr cell strain is cultivated in Gamborg medium [85] supplemented with 30 g L^{-1} sucrose and 1 mg L^{-1} 2,4-dichlorophenoxyacetic acid at pH is 6.0 and subcultured every 2 weeks [4]. For both strains, initial density is close to 30 g L^{-1} and medium is sterilized by autoclave (30 min at 115 °C).

The stirred-tank bioreactor is a 14-L vessel (New Brunswick Scientific, USA) equipped with a pitched blade impeller (10 L working volume). The bioreactor containing 9 L of fresh medium is autoclaved for 40 min at 121 °C. Dissolved oxygen is maintained at 30% by increasing or decreasing airflow rate. The bioreactor is equipped with a sterilisable oxygen probe (lnPro 6110, Ingold Mettler Toledo GmbH, Switzerland), and a mass flowmeter. The stirrer speed is adjusted at 100 rpm. For cultures in WU bioreactors (10, 20, 30 and 100 L working volumes) and SB bioreactors (10, 20, 50 and 70 L working volumes), medium addition, agitation and aeration have been described earlier.

In all systems, inoculation is performed as follows: 14-day-old soya cells or 7-day-old tobacco cells are aseptically transferred from Erlenmeyer flasks or from the stirred-tank bioreactor (for the WU and SB bioreactors) to the bioreactor via a sterile container. The inoculum is prepared in order to reach circa 30 g L^{-1} fresh weight in the inoculated bioreactor.

Cell doubling time (td) is defined by the expression: td = ln2/μ, where (μ), the apparent growth rate, is calculated as:

$$\mu = \frac{\ln (\textit{final DW / initial DW})}{\Delta t} \quad \text{during exponential growth phase.}$$

Extraction and analysis of isoflavones are described in [8].

3.3.2   Results

Tobacco Cell Culture

Figures 8 and 9 show an example of the growth kinetics currently obtained in the different culture systems. Tobacco cells were cultivated in four different volumes in the WU bioreactor and in the SB bioreactor. The results obtained were similar to
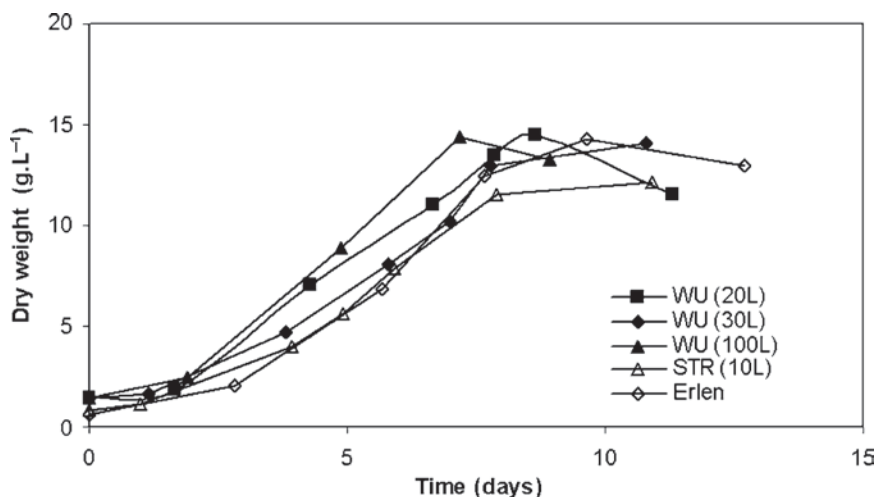

Fig. 8 Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor and WU bioreactor (reproduced from [8])
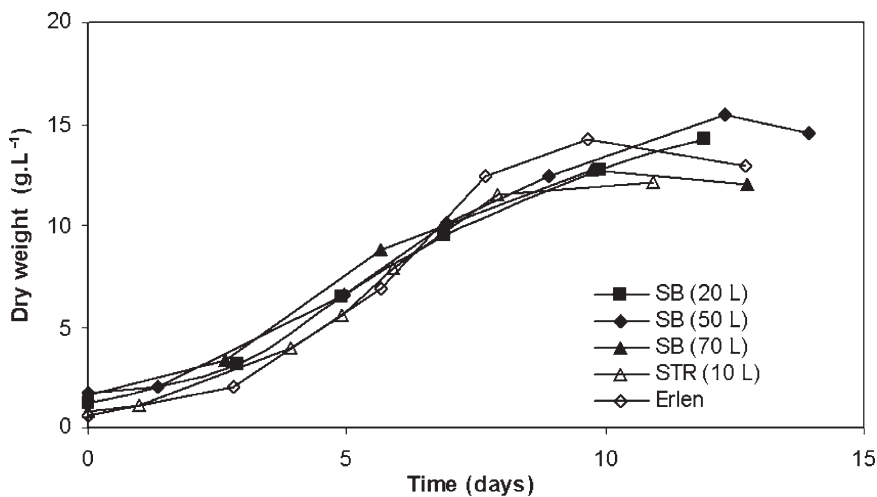

Fig. 9 Growth of tobacco cell cultures in Erlenmeyer flasks, stirred-tank bioreactor and SB bioreactor (reproduced from [8])

Table 5 Growth parameters of tobacco and soya cell cultures in Erlenmeyer flasks, 10-L stirred-tank, *WU* and SB bioreactors

| Type of system (working volume) | Tobacco cells | | | Soya cells | | |
	Number of independent experiments	Max dry weight (g L^{-1})	Doubling time (days)	Number of independent experiments	Max dry weight (g L^{-1})	Doubling time (days)
Erlenmeyer flask[a]	3	13.9 ± 0.5	2.1 ± 0.4	3	14.6 ± 0.4	2.8 ± 0.6
STR (10 L)[b]	3	14.4 ± 2.1	1.9 ± 0.3	3	12.9 ± 4.7	3.5 ± 1.5
WU (10 L)	2	13.6 ± 0.0	2.3 ± 0.1	1	14.3	2.5
WU (20 L)	4	12.8 ± 1.9	2.3 ± 0.4	5	13.8 ± 1.8	3.2 ± 0.7
WU (30 L)	3	12.6 ± 1.3	2.0 ± 0.2	2	16.5 ± 0.5	2.4 ± 0.2
WU (100 L)	5	13.0 ± 1.1	2.3 ± 0.3	2	15.5 ± 0.1	2.2 ± 0
SB (10 L)	2	17.2 ± 0.6	2.0 ± 0.2	–	–	–
SB (20 L)	5	13.7 ± 0.6	2.1 ± 0.2	6	13.9 ± 0.9	2.8 ± 0.3
SB (50 L)	3	14.2 ± 1.2	2.0 ± 0.4	3	14.7 ± 2.0	2.7 ± 0.5
SB (70 L)	2	12.9 ± 0.3	2.4 ± 0.1	–	–	–

[a]Erlenmeyer flask: 250 mL with 100 mL medium, 100 rpm on a gyratory shaker. 26 °C
[b]10-L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 VVM, 26 °C

Table 6 Isoflavone production

Type of system (working volume)	Number of experiments	Max. Isoflavone concentration (mg g^{-1} DW)
Erlenmeyer flask[a]	6	61 ± 35
Stirred-tank bioreactor (10 L)[b]	3	28[NS] ± 20
WU (20 L)	5	39[NS] ± 39
SB (20 L)	6	23* ± 11
SB (50 L)	3	48[NS] ± 34

[a]Erlenmeyer flask: 250 mL with 100 mL medium, 100 rpm on a gyratory shaker. 26 °C
[b]10-L stirred-tank bioreactor: New Brunswick bioreactor, 100 rpm, 0.25–0.5 VVM, 26 °C
ANOVA: *NS*: nonsignificantly different from Erlenmeyer flasks*:significantly different from Erlenmeyer flasks; $p < 0.05$

those observed in traditional systems (Erlenmeyer flasks and bioreactor). This was confirmed using data collected from several independent experiments (Table 5). These results establish that cultivation in WU bioreactor with a 100 L working volume can be used instead of a traditional stainless steel stirred tank bioreactor. For the SB bioreactor, cultivation up to 70 L working volume has been demonstrated.

Soya Cell Culture and Isoflavone Production

The results are different from those obtained with tobacco cells (see Table 4): the stirred tank bioreactor is the least efficient culture system. Soya cell suspensions are much more aggregated (clump formation) than tobacco suspension. This result could be due to a higher shear stress sensitivity of the cells due to mechanical impeller agitation. The cultivation in WU and SB bioreactors is similar to cultivation in Erlenmeyer flasks.

Isoflavone production has been measured in three systems (20-L WU and SB bioreactors, and 50-L SB bioreactors), in comparison with Erlenmeyer flasks and stirred-tank bioreactor (Table 6). A large variability is observed between experiments, whatever the culture system, showing that the optimal control of the culture conditions for isoflavone production is not reached in any culture system. Statistical analysis (ANOVA) confirms that the culture systems are not significantly different from the Erlenmeyer flasks, except the SB bioreactor (20-L scale) which appears to give lower concentrations. New culture systems are not detrimental to the production of isoflavones, but further investigation is required to identify the key parameters linked to the biosynthesis and accumulation of isoflavones.

3.4 Scale-Up; Advantages and Limitations

3.4.1 Scale-Up and Volume Increase

Studies are still on-going to scale-up the systems, that is increase the working volumes while maintaining similar growth conditions.

The WU systems are geometrically similar (constant bag length over width ratio) with a filling level ranging from 33 to 50%; for a given size, increasing the filling level favours the system compactness but also impacts the wave formation or quality and therefore the growth conditions. For the SB bioreactor, the most crucial parameter is the column diameter; a small increase deeply impacts the total volume but is also, at the same time, truly detrimental to bubble formation and therefore to mixing and oxygenation.

Besides traditional scale-up as described above, both systems also present another form of scaling-up. For large culture volumes, the WU bioreactor can be adapted to grow the inoculum and the batch in the same bag (Fig. 10). The bioreactor
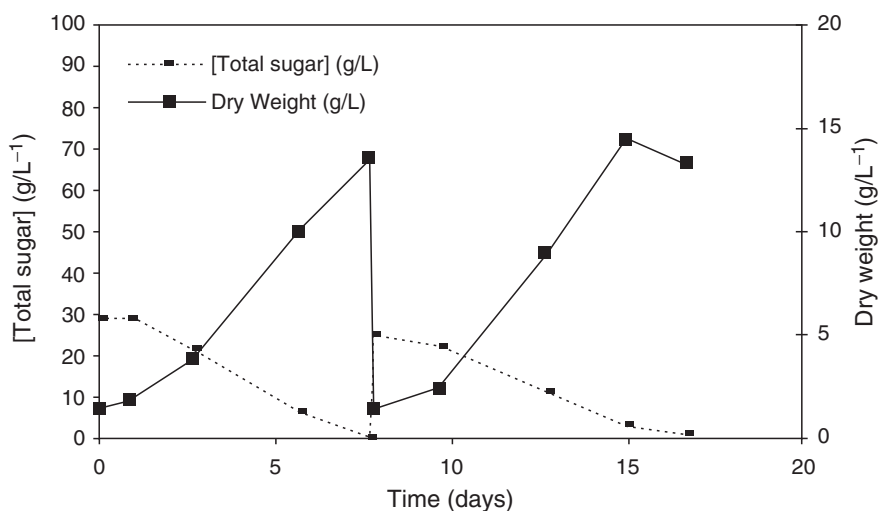

Fig. 10 Growth of tobacco cell culture in two successive steps (10 and 100 L working volumes) in a 200-L WU bioreactor (reproduced from [8])

is partitioned during the first part of the culture (smaller surface area) before going back to its initial shape when medium is added to grow the entire batch. This technique can reduce the risks of contamination through successive inoculations and decrease scale up time. Nevertheless, the difference of several parameters (headspace volume, filling level, k_La, etc.) from one phase to the other has to be studied to validate this improvement. For the SB bioreactor, running multiple experiments in parallel or increasing production volumes can be rapidly achieved having several culture systems close together (or even connected) in a small area.

3.4.2   Advantages and Limitations

The disposable systems described here offer many benefits and practical advantages in comparison with traditional systems, especially lightness and versatility and they also permit new designs (that would have been impossible in glass or stainless steel, for example the WU system). The new design (and the novel agitation mode) also implies new engineering studies to prove at least the innocuity, at best the added value of these novel systems in comparison with well known and trustable ancient systems, especially if these bioreactors are to be used in the biopharmaceutical industry. Improved or simplified designs finally mean less or no maintenance and minimal needs for cleaning. Scale up is simplified and faster, up to a certain limit, since flexible containers will not be able to hold large volumes without any support. Working with disposable bioreactors instead of re-usable ones also implies to trust and validate the manufacturer and/or the manufacturing process (whether the systems are home-made or contract-manufactured) since each bioreactor is a novel process unit.

4   Conclusion

There is a consensus according to which temporary immersion cultures will play a dominant role on the future of plant micropropagation but ideal commercial equipments have yet to be invented [86]. Particularly, it is a promising way to easily optimize light illumination inside bulk cultures of plant tissues. So far, the various illuminated bioreactors designed to introduce light to the cultures through optical fibre are not yet efficient [45]. Nevertheless, one of the key points is the quality of ports and welds: for a commercial implementation, this material must be manufactured by plastic specialists. Scale-up of micropropagation may probably be facilitated by the commercialization of large and adapted plastic bags. The box-in-bag bioreactor can be an example of such innovations and a promising technology even if it is too early to say if such a disposable device could be applied for other plant species or for the micropropagation from shoots or other organogenic tissues.

  The use of disposables is slowly but steadily increasing in the field of cell culture where their advantages for the manufacturing of biopharmaceuticals are well-known and largely agreed on [87]:

– Simplified facility design
– Greater flexibility for small to medium scale operations
– Modular manufacturing done in a 'rapid factory' based on disposable, pre-validated units that can be deployed very quickly
– Producing otherwise uneconomical drug candidates
– Possible multi-product facilities: multi-product manufacturing in one suite using disposables will allow high capacity utilization
– Disposables minimize cross contamination (cell therapy procedures)

The present chapter underlines the interest of developing disposable plastic-based systems with two different applications in the field of plant biotechnology: small to medium scale plant cell cultures can be easily obtained for biomass, metabolites or recombinant proteins production; for plant propagation, the system we have developed is, to our knowledge, the first one allowing the routine production of millions of coffee plantlets each year.

References

1. Pétiard V, Courtois D, Masseret M, Delaunay P, Florin D (1987) Existing limitations and potential applications of plant cells cultures. In: Neijssel OM, van der Meer RR, Luyben K (eds) Proceedings 4th European Congress on Biotechnology, Elsevier, Amsterdam, p 157
2. Courtois D, Yvernel D, Florin B, Pétiard V (1988) Phytochemistry 27:3137
3. Verdelhan des Molles D, Gomord V, Bastin M, Faye L, Courtois D (1999) J Biosci Bioeng 87:302
4. Federici E, Touche A, Choquart S, Avanti O, Fay L, Offord E, Courtois D (2003) Phytochemistry 64:717
5. Girard LS, Bastin M, Courtois D (2004) Plant Cell Tissue Org Cult 78:253
6. Girard LS, Fabis Y, Bastin M, Courtois D, Pétiard V, Koprowski H (2006) Biochem Biophys Res Commun 345:602
7. Sohier S, Courtois D (2007) *Ginkgo biloba* and production of secondary metabolites. In: Kayser O, Quax W (eds) Medical plant biotechnology: from basic research to industrial applications, vol 2. Wiley-VCH, Weinheim, p 493
8. Terrier B, Courtois C, Hénault N, Cuvier A, Bastin M, Aknin A, Dubreuil J, Pétiard V (2007) Biotechnol Bioeng 96:914
9. Zamarripa A, Ducos JP, Bollon H, Dufour M, Pétiard V (1991) Café Cacao 35:233
10. Zamarripa A, Ducos JP, Tessereau H, Bollon H, Eskès AB, Pétiard V (1991) Developpement d'un procédé de multiplication en masse du caféier par embryogenèse somatique en milieu liquide. In: ASIC (ed) Proceeding of 14th Colloquium of International Coffee Science Association. ASIC, Vevey, p 392
11. Ducos JP, Bollon H, Pétiard V (1993) Appl Microbiol Biotechnol 39:465
12. Ducos JP, Zamarripa A, Eskes A, Pétiard V (1993) Production of somatic embryos of coffee in a bioreactor. In: ASIC (ed) Proceeding of 15th Colloquium of International Coffee Science Association. ASIC, Vevey, p 89
13. Jehan H, Courtois D, Ehret C, Lerch K, Pétiard V (1994) Plant Cell Rep 13:671
14. Ducos JP, Gianforcaro M, Florin B, Pétiard V, Deshayes A (1999) A technically and economically attractive way to propagate elite *Coffee canephora* (Robusta) clones: *in vitro* somatic embryogenesis. In: ASIC (ed) Proceeding of 18th Colloquium of International Coffee Science Association. ASIC, Vevey, p 295

15. Ducos JP, Alenton R, Reano JF, Kanchanomai C, Deshayes A, Pétiard V (2003) Euphytica 131:215
16. Ducos JP, Chantanumat P, Vuong P, Lambot C, Pétiard V (2007) Acta Hortic 764:33
17. Ducos JP, Lambot C, Pétiard V (2007) Int J Plant Dev Biol 1:1
18. Ducos JP, Labbé G, Lambot C, Pétiard V (2007) In Vitro Cell Dev Bio-Plant 43:652
19. Ducos JP, Prevot A, Lambot C, Pétiard V (2007) Acta Hortic In press
20. Ammirato PV, Styer DJ (1985) Strategies for large scale manipulation of somatic embryos in suspension cultures. In: Zaitlin M, Day P, Hollaender A (eds) Biotechnology in plant science: relevance to agriculture in the eighties. Academic Press, New York, p 161
21. Ziv M (1991) Isr J Bot 40:145
22. Takayama S, Akita M (1994) Plant Cell Tissue Org Cult 39:147
23. Tisserat B, Vandercook CE (1985) Plant Cell Tissue Org Cult 5:107
24. Aitken-Christie J, Jones C (1987) Plant Cell Tissue Org Cult 8:185
25. Krueger S, Robacker C, Simonton W (1991) Plant Cell Tissue Org Cult 27:219
26. Simonton W, Robacker C, Krueger S (1991) Plant Cell Tissue Org Cult 27:211
27. Hvoslef-Eide AK, Preil W (2005) Liquid culture systems for in vitro plant Propagation. Springer, Dordrecht
28. Dutta Gupta S, Ibaraki Y (2006) Plant tissue culture engineering. Springer, Dordrecht
29. Misawa M (1994) Plant tissue culture: an alternative for production of useful metabolites. FA0 Agricultural Services Bulletin No 108. Food and Agriculture Organization of the United Nations, Rome
30. Mulabagal V, Tsay H (2004) Int J Appl Sci Eng 2:29
31. Takahashi S, Fujita Y (1991) Production of shikonin. In: Komamine A, Misawa M, Di-Cosmo F (eds) Plant cell culture in Japan. CMC, Tokyo, p 72
32. Hibino K, Ushiyama K (1999) Commercial production of ginseng by plant tissue culture technology. In: Fu TJ, Singh G, Curtis WR (eds) Plant cell and tissue culture for the production of food ingredients. Kluwer, New York, p 215
33. Matsubara K, Fujita S (1991) Production of berberine. In: Komamine A, Misawa M, Di Cosmo F (eds) Plant cell culture in Japan. CMC, Tokyo, p 39
34. Venkat K (1998) Pure Appl Chem 70:2127
35. Hellwig S, Drossard J, Twyman RM, Fischer R (2004) Nat Biotechnol 22:1415
36. Choi SM, Lee O, Kwon S, Kwak SS, Yu D, Lee HS (2003) Biotechnol Lett 25:213
37. Gao J, Hooker BS, Anderson DB (2004) Prot Exp Purif 37:89
38. McDonald KA, Hong LM, Trombly DM, Xie Q, Jackman AP (2005) Biotechnol Prog 21:728
39. Soderquist RG, Lee JM (2005) Plant Cell Rep 24:127
40. Sorrentino A, Schilberg S, Fischer R, Rao R, Porta R, Mariniello L (2005) Int J Biochem Cell Biol 37:852
41. Shaaltiel Y, Bartfeld D, Hashmueli S, Baum G, Brill-Almon E, Galili G, Dym O, Boldin-Adamsky SA, Silman I, Sussman JL, Futerman AH, Aviezer D (2007) Plant Biotechnol J 5:579
42. Peebles CAM, Gibson SI, Shanks JV, San KY (2007) Biotechnol Prog 23:1517
43. Warzecha H, Frank A, Peer M, Gillam EMJ, Guengerich FP, Unger M (2007) Plant Biotechnol J 5:185
44. Yoshioka T, Fujita Y (1988) Economic aspects of plant cell biotechnology. In: Pais MS, Mavituna F, Novais JM (eds) Plant cell biotechnology. Springer, Berlin, p 475
45. Takayama S, Akita M (2006) Bioengineering aspects of bioreactor in plant propagation. In: Dutta Gupta S, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, p 83
46. Weathers PJ, Giles K (1988) In Vitro Cell Dev Bio-Plant 24:727
47. Adelberg J (2004) In Vitro Cell Dev Bio-Plant 40:245
48. Adelberg J (2006) Agitated, thin-films of liquid medium for efficient micropropagation. In: Dutta Gupta S, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, p 101
49. Alvard D, Cote F, Teisson C (1993) Plant Cell Tissue Org Cult 32:55
50. Berthouly M, Dufour M, Alvard D, Carasco C, Alemano L, Teisson C (1995) Coffee micropropagation in a liquid medium using the temporary immersion technique. In: ASIC (ed) Proceeding of 16th Colloquium of International Coffee Science Association. ASIC, Vevey, p 514

51. Etienne H, Bertrand B, Anthony F, Cote F, Berthouly M (1997) L'embryogenèse somatique: un outil pour l'amélioration genetique du caféier. In: ASIC (ed) Proceeding of 17th Colloquium of International Coffee Science Association. ASIC, Vevey, p 457

52. Akula A, Becker D, Bateson M (2000) Plant Cell Rep 19:1140

53. Alister BM, Finnie J, Watt MP, Blakeway F (2005) Use of temporary immersion system (RITA®) for production of commercial *Eucalyptus* clones in Mondi Forests (SA). In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 425

54. Escalona M, Lorenzo JC, Gonzales B, Daquinta M, Gonzales JL, Desjardins Y, Borotto CG (1999) Plant Cell Rep 18:743

55. Jimenez-Gonzales E (2005) Mass propagation of tropical crops in temporary immersion systems. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 197

56. Hempfling T, Preil W (2005) Application of a temporary immersion system in mass propagation of *Phalaenopsis*. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 231

57. Paek KY, Chakrabarty D, Hahn EJ (2005) Application of bioreactor systems for large scale production of horticultural and medicinal plant. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 96

58. Damiano C, La Starza SR, Monticelli S, Gentille A, Carboni E, Frattarelli A (2005) Propagation of *Prunus* and *Malus* by temporary immersion. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 243

59. Gontier E, Piutti S, Gravot A, Milesi S, Grabner A, Massot B, Lievre K, Tran M, Goergen JL, Bourgaud F (2005) Development and validation of an efficient low cost bioreactor for furanocoumarin production with *Ruta graveolens* shoot cultures. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 509

60. Wilken D, Jiménez Gonzàlez E, Hohe A, Jordan M, Gomez Kosky R, Hirshmann G, Gerth A (2005) Comparison of secondary plant metabolite production in cell suspension, callus culture and temporary immersion system. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 525

61. Etienne H, Berthouly M (2002) Plant Cell Tissue Org Cult 69:215

62. Savangikar VA, Savangikar C, Daga RS, Pathak S (2005) Potentials for cost reduction in a new model of commercial micropropagation. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 403

63. Tanaka M, Jinno K, Goi M, Higashiura T (1988) Acta Hortic 230:73

64. Ziv M, Ronen G, Raviv M (1998) Proliferation of meristematic clusters in disposable presterilized plastic bioreactors for the large-scale micropropagation of plants. In Vitro Cell Dev Bio-Plant 34:152

65. Ziv M (2005) Simple bioreactor for mass propagation of plants. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 79

66. Levin R, Tanny G (2004) Bioreactors as a low cost option for tissue culture. In: IAEA (ed) Low cost options for tissue culture technology in developing countries. IAEA, Vienna, p 47

67. Sluis CJ (2006) Integrating automation technologies with commercial micropropagation. In: Dutta Gupta S, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, p 231

68. Eibl R, Eibl D (2006) Design and use of the wave bioreactor for plant cell culture. In: Dutta Gupta S, Ibaraki Y (eds) Plant tissue culture engineering. Dordrecht, Springer, p 203

69. Etienne-Barry D, Bertrand B, Vasquez N, Etienne H (1999) Plant Cell Rep 19:111

70. Sanpote S, Ducos JP, Lambot C, Kunasol T, Kasinkasaempong K, Chantanumat P, Pétiard V (2006) First industrial massive propagation of *Coffea canephora* through somatic embrygenesis organized in Thailand. In: ASIC (ed) Proceeding of 21th Colloquium of International Coffee Science Association. ASIC, Vevey, In press

71. Kosai T, Kubota C, Jeong BR (1997) Plant Cell Tissue Org Cult 51:49

72. Kosai T, Xiao Y (2006) A commercialized photoautotrophic micropropagation system. In: Dutta Gupta S, Ibaraki Y (eds) Plant tissue culture engineering. Springer, Dordrecht, p 355

73. Afreen F, Zobayed SMA, Kozai T (2002) Ann Bot 90:20
74. Zobayed SM, Afreen F, Xiao Y, Kozai T (2004) In Vitro Cell Dev Bio-Plant 40:450
75. Singh V (1999) Cytotechnology 30:149
76. Hsiao TY, Bacani FT, Carvahlo EB, Curtis WR (1999) Biotechnol Prog 15:114
77. Curtis WR (1999) Achieving economic feasibility for moderate-value food and flavor additives: a perspective on productivity and proposal for production technology cost reduction. In: Fu TJ, Singh G, Curtis WR (eds) Plant cell culture for the production of food ingredients. Kluwer, New York, p 225
78. Palazon J, Mallol A, Eibl R, Lettenbauer C, Cusidó RM, Piñol MT (2003) Planta Med 69:344
79. Bentebibel S, Moyano E, Palazon J, Cusidó RM, Bonfill M, Eibl R, Piñol MT (2005) Biotechnol Bioeng 89:647
80. Davies RM, Taylor G (1950) Proc R Soc A London 200:375
81. Nicklin DJ, Wilkes JO, Davidson JF (1962) Trans Inst Chem Engrs 40:61
82. Sousa RG, Riethmuller ML, Pinto AMFR, Campos JBLM (2005) Chem Eng Sci 60:1589
83. Nagata T (2004) When I encountered tobacco BY-2 cells! In: Nagata T, Hasezawa S, Inzé D (eds) Tobacco BY-2 cells. Biotechnology in agriculture and forestry, vol 53. Springer, Berlin, p 1
84. Murashige RS, Skoog F (1962) Physiol Plant 67:603
85. Gamborg OL, Miller RA, Ojim K (1968) Ex. Cell Res 50:151
86. Preil W (2005) General introduction: a personal reflection on the use of liquid media for in vitro culture. In: Hvoslef-Eide AK, Preil W (eds) Liquid culture systems for in vitro plant propagation. Springer, Dordrecht, p 1
87. Langer ES, Price BJ (2007) BioPharm Int 48–56

Transport Advances in Disposable Bioreactors for Liver Tissue Engineering

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Abstract Acute liver failure (ALF) is a devastating diagnosis with an overall survival of approximately 60%. Liver transplantation is the therapy of choice for ALF patients but is limited by the scarce availability of donor organs. The prognosis of ALF patients may improve if essential liver functions are restored during liver failure by means of auxiliary methods because liver tissue has the capability to regenerate and heal. Bioartificial liver (BAL) approaches use liver tissue or cells to provide ALF patients with liver-specific metabolism and synthesis products necessary to relieve some of the symptoms and to promote liver tissue regeneration. The most promising BAL treatments are based on the culture of tissue engineered (TE) liver constructs, with mature liver cells or cells that may differentiate into hepatocytes to perform liver-specific functions, in disposable continuous-flow bioreactors. In fact, adult hepatocytes perform all essential liver functions. Clinical evaluations of the proposed BALs show that they are safe but have not clearly proven the efficacy of treatment as compared to standard supportive treatments. Ambiguous clinical results, the time loss of cellular activity during treatment, and the presence of a necrotic core in the cell compartment of many bioreactors suggest that improvement of transport of nutrients, and metabolic wastes and products to or from the cells in the bioreactor is critical for the development of therapeutically

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effective BALs. In this chapter, advanced strategies that have been proposed over to improve mass transport in the bioreactors at the core of a BAL for the treatment of ALF patients are reviewed.

Keywords Bioartificial, Bioreactor, Cell, Disposable, Liver, Mass, Transport.

Contents

1 Introduction

Acute liver failure (ALF) is a devastating diagnosis. Overall survival associated with best medical care has improved from approximately 20% in the 1970s to approximately 60% during the past decade [1]. Survival is etiology dependent, ranging from ~25% for drug-induced ALF, hepatitis B, and cryptogenic cases to ~60% for acetaminophen overdose, hepatitis A, and ischemia [2]. Liver transplantation (LTx), with 1-year survival rates of 60–80% for ALF patients, is the therapy of choice for ALF patients [3, 4]. Availability of organs for LTx is problematic: 6,530 patients out of 17,298 on the wait list received a liver transplant (38%) in the United States in 2006–2007 (www.ustransplant.org, 2008). Etiologies and transplant rates vary from country to country [5, 6], but reflect similar trends. In many countries, the high social costs of transplantation and the associated year-long immunosuppressive therapy also pose serious ethical questions on the eligibility criteria for liver transplantation and often further limit the number of LTx procedures [7].

The prognosis of many of ALF patients may improve without the need for LTx if essential liver functions are restored during liver failure by means of auxiliary methods [1, 2, 8]. In fact, liver tissue has the capability to regenerate and heal [9]. In the last decade, exploitation of this possibility has led to the development of innovative treatments for ALF that include split-liver transplantation, extracorporeal artificial liver (AL) support (nonbiological detoxification), extracorporeal bioartificial liver (BAL) support (cell-based systems), and in vivo tissue or cell transplantation [10]. Success of split-liver transplantation has been reported to be close to that of LTx, but the procedure is not broadly performed and surgery may be complicated by anatomical variations [11].

Extracorporeal AL approaches are directed toward removal of plasma toxins (e.g., ammonia, mercaptans, free phenols, bile acids, benzodiazepines, etc.) that accumulate in ALF patients [12]. To this purpose, hemodialysis, hemoperfusion or

plasmapheresis cartridges and procedures have been used, as clinically available or modified by using albumin (dissolved in the stripping solution or adsorbed in the pores of asymmetric membranes) to facilitate removal of protein-bound toxins [13–18]. However, AL approaches have not yet demonstrated significant improvement over conventional patient management.

BAL approaches use liver tissue or cells to provide ALF patients with liver-specific metabolism and synthesis products necessary to relieve some of the symptoms (e.g., cerebral oedema and bleeding) to promote liver tissue regeneration and, ideally, to provide the whole set of liver-specific biosynthetic and biotransformation functions that the failing liver cannot provide. BAL treatments based on direct perfusion of xenogeneic whole liver or liver slices, or cross-hemodialysis against xenogeneic livers have been reported to have some beneficial effects, but are impractical in the clinical setting [19]. Such xenogeneic approaches also put the ALF patient at risk of massive immune reaction against the xenogeneic organ or released soluble antigens.

The most promising BAL treatments are based on the use of mature liver cells (e.g., primary or immortalized) or cells that may differentiate into hepatocytes (e.g., adult stem cells or progenitor cells) to perform liver-specific functions [20]. In fact, adult hepatocytes have been shown to perform all essential liver functions [21, 22]. Unfortunately, in vitro isolated primary hepatocytes cultured in suspension lose their morphology, depolarize, dedifferentiate, are not able to perform the natural liver functions, and die within a few hours. Even in in vitro cultures that have been shown to stabilize their functions for a few weeks, isolated hepatocytes do not generally proliferate. Available information on the structure–function relationships of healthy and pathological liver tissue is also incomplete.

The challenge in BAL and in vivo tissue transplantation for treatment of ALF patients is design and development of liver tissue patterned after the native liver microarchitecture so as to foster the same cellular and functional relationships that exist in the healthy natural liver. The formidable technical challenge of engineering liver tissue in vitro is reflected in the multicellular and highly hierarchical architecture of the natural liver tissue, with complex vascularization, and the mass of the biological substitute presumed necessary to restore homeostasis in ALF patients. Hepatectomy studies suggest that a tissue engineered (TE) liver construct for effective treatment of ALF needs to perform metabolic functions equivalent to about 10–30% of the natural liver mass for an estimated 200–500 g mass of metabolically active parenchymal liver cells for an adult human [9, 23].

To date, BAL treatments for ALF that have been clinically evaluated are based on TE liver tissue containing natural or transformed liver cells seeded on two- (2D) or three-dimensional (3D) nonresorbable scaffolds that provide the template for cell adhesion, reorganization, proliferation (transformed liver cells), and differentiation. The BAL-TE liver construct is generally cultured in continuous-flow bioreactors that treat the patient's blood or plasma flowing in an extracorporeal loop. Cell sourcing, development, design, operational features, preclinical and clinical performance of the bioreactors and the BALs proposed over the years have been presented and discussed in many review papers [7, 22, 24–30].

Bioreactors that have been proposed as the core of BAL systems testify to the ingenuity of researchers active in the field. Listed in Table 1, the BAL systems differ

Table 1 Continuous-flow bioreactors used for the clinical treatment of acute liver failure (ALF) patients (adapted from [28])

Bioreactor brand name and type	Membrane type and NMWCO	Auxiliary physical treatment	Perfusate and flow rate	Cell number and type	References
Excorp BLSS® Shell-and-tube HF membrane bioreactor with cells in the shell embedded in collagen outside a bundle of membranes and blood flowing in the membrane lumen	Polysulphone 100 kDa	Blood oxygenator	Blood	8–20 × 10^9 (ca. 100 g) porcine hepatocytes	[31, 32]
Vital Therapies ELAD® Shell-and-tube HF membrane bioreactor with cells adherent on the external membrane surface and blood fed to membrane lumen	Cellulose acetate 70 kDa	Blood oxygenator	Blood 150–300 mL min^{-1}	2 × 10^10 (ca. 200 g) cells of human epatoblastoma C3A line	[33]
Arbios Systems HepatAssist® Shell-and-tube HF membrane bioreactor with cryopreserved cell clumps adherent on dextran microbeads in the shell and plasma fed to membrane lumen	Microporous polysulphone maximal pore size 0.2 μm	Activated charcoal adsorbent cartridge	Plasma-recirculated at 400 mL min^{-1}	4–6 × 10^9 (ca. 50 g) cryopreserved porcine hepatocytes	[34]
MELS CellModule Interwven four compartment membrane network bioreactor for 3D cell perfusion at tissue densities with repeating units consisting of two overlaid hydrophilic microporous HF membrane mats and a mat of microporous hydrophobic membranes interposed among them for oxygen supply. Cells cultured outside and among the membranes are perfused by	Microporous hydrophilic polyethersulphone pore size 0.2 μm Hydrophobic asymmetric polymethylpentene for plasma oxygenation maximal pore size 0.1 μm	Dialysis and removal of hydrophobic toxins with a concentrated albumin solution possible	Plasma in single pass at 40–60 mL per minor or Plasma recirculated at 400 mL min^{-1}	2–8 × 10^10 (ca. 500 g) porcine or human liver cells	[17, 35, 36]
AMC-BAL Cylindrical packed bioreactor with cell-seeded spiral wound polyester nonwoven fabric with HF membranes for oxygen supply, and plasma axially perfusing the fabric	Hydrophobic symmetric polypropylene for plasma oxygenation maximal pore size 0.1 μm		Plasma	2 × 10^9 porcine cells	[37]
RAnD BAL Annular packed bioreactor with cell seeded spiral wound polyester nonwoven fabric, and plasma radially perfusing the fabric		Bilirubin adsorption cartridge	Plasma	2–2.3 × 10^10 porcine hepatocytes	[38]

in the type and mass of cells used, the geometrical and physical–chemical properties of the scaffolds, the cell seeding technique, the bioreactor design and operation, the fluid treated (blood vs plasma), and the possible use of auxiliary devices for the physical treatment of the processed fluid (e.g., to reduce the toxin load on the liver cells or to replenish oxygen in the fluid entering the bioreactor, etc.). Laboratory tests and trials with animal models of ALF have generally shown that BALs are promising alternatives to LTx in the treatment of ALF. A few of the "first generation" BALs have undergone extensive experimental evaluation and are still being tested in the clinical setting but, to date, none of the proposed BALs has yet been approved for clinical treatment of ALF or chronic liver failure.

Five out of the six BAL systems that have been clinically evaluated use primary porcine hepatocytes (Arbios HepatAssist®, MELS CellModule, Excorp BLSS, AMC-BAL, and RAnD BAL). The Vital Therapies ELAD® uses the tumor-derived C3A cell line, a subclone of the HepG2 cell line. In four out of six bioreactors cells are seeded outside perm-selective hollow fiber membranes (in the extracapillary space of the bioreactor), with whole blood or plasma flowing in the membrane lumen (Arbios HepatAssist®, MELS CellModule, Excorp BLSS, Vital Therapies ELAD®). In two of them (AMC-BAL and RAnD BAL), cells are cultured in aggregates attached to a nonwoven polyester fabric and are directly perfused with plasma.

Clinical evaluations have generally shown that treatments based on these BALs are safe and have shown that immunological reactions, zoonosis and tumorigenicity were not a problem for the patient [34, 36, 39–43]. The reported clinical studies have shown that patients may be successfully bridged to LTx with BAL-based treatents using TE liver constructs, but have not clearly proven the efficacy of treatment as compared to standard supportive treatments. The largest scale, prospective, multicenter, randomized phase II/III trial of the HepatAssist® BAL, a "first generation BAL," did show a statistically significant higher survival rate compared to controls receiving state-of-the-art standard supportive treatment, but only for patients with fulminant and subfulminant hepatic failure and only after accounting for the effect of the different etiology of ALF and liver transplantation on patients' survival [34]. Such ambiguous clinical results, the time loss of cellular activity during treatment, and the presence of a necrotic core in the cell compartment of many bioreactors at the end of treatment suggest that improvement of transport of nutrients, and metabolic wastes and products to or from the cells in the bioreactor is critical for the development of therapeutically effective BALs [44].

The knowledge and experience gained from the experimental activity in BAL development performed in the laboratory, in animal models of ALF, and in clinical evaluations indicate that the ideal bioreactor for a BAL ought to: provide cells with nutrients and oxygen; remove carbon dioxide and waste metabolites to prevent cell death; provide cells with biochemical and physical cues that foster cell reorganization into liver-like aggregates and cell differentiation; preserve the liver cell phenotype for the treatment time; prevent cell rejection (if allo- or xenogeneic cells are used) and intoxication caused by the ALF plasma; promote the unhindered transport of liver-specific metabolic products into the blood stream of the patient; and be operated so as to maximize the BAL therapeutic efficacy. Review papers are available

in literature discussing the effect of liver cell coculture, culture conditions and techniques, and cell scaffolds on bioreactor performance. In this chapter, advanced strategies that have been proposed over to improve mass transport in the bioreactors at the core of a BAL for the treatment of ALF patients are reviewed.

2 Strategies to Improve Mass Transport in BAL Bioreactors

Ensuring proper transport of essential nutrients (oxygen, sugars, amino acids, etc.) to the cells and waste metabolites (CO_2, lactate, etc.) and liver-specific metabolic products (clotting factors, growth factors, etc.) away from the cells in a TE liver construct is essential for proper bioreactor performance. Transport is, however, complicated by the high cell density typical of the natural liver tissue, typically low concentrations of nutrients, and sensitivity of liver cells to waste metabolites [26, 45–48]. Poor oxygen and glucose supply has been correlated with necrotic regions in tumors and in dense cell aggregates [49–52]. Low oxygen concentrations have been reported to affect cell viability and function [26]. Nonuniform spatial distributions of nutrients, metabolic wastes and products may have important effects on cell phenotype, motility and survival, on the bioreactor performance, and on the therapeutic efficacy of the treatment as a whole.

Conceptually, BAL bioreactors are heterogeneous (i.e., more than one phase is present) and may be thought of as being comprised of geometrically and volumetrically distinct compartments that intercommunicate through mass exchange. Membranes are often used to separate compartments in a bioreactor and their finite volume also defines them as a compartment. In fact, phenomena occurring in the membrane wall significantly affect mass exchange and overall efficacy of the bioreactor. In spite of this, BAL bioreactors are commonly classified without accounting for the membrane as a compartment – a convention we also adopt for continuity with prior literature. However, because of their importance in bioreactor performance, membranes are discussed in great detail in Sects. 2.1 and 2.3.

The RAnD BAL is a two-compartment bioreactor that radially perfuses oxygenated nutrient media (plasma in clinical operation) through a nonwoven mesh scaffold (the plasma compartment) containing adherent hepatocyte aggregates (the cell compartment). The AMC-BAL is distinguished from the RAnD BAL by axial flow rather than radial flow perfusion through a nonwoven polyester mesh scaffold containing adherent hepatocyte aggregates and a third compartment consisting of the lumen of axially aligned hydrophobic oxygenation fibers used to provide local, integral oxygen to the cells. The Arbios HepatAssist®, Excorp BLSS, and Vital Therapies ELAD® use two compartment bioreactors in which nutrient media (blood or plasma in clinical operation) flows through the lumen of hydrophilic hollow fiber membranes (first compartment) with cells housed in a second compartment external to the hollow fibers. The MELS CellModule is a four-compartment bioreactor with two independent compartments in the lumen of two distinct capillary systems bounded by hydrophilic hollow fiber membranes for nutrient media

perfusion (plasma in clinical operation), a third compartment consisting of the lumen of hydrophobic oxygenation fibers used to provide local, integral oxygen to the cells, and a fourth compartment, external to the three fiber lumen compartments, that houses cells. The hollow fiber membrane mats for media and oxygen perfusion are interposed to achieve decentralized mass exchange with low concentration gradients and scalability of the bioreactor size. The two sets of capillary media perfusion fibers can be operated in counter-directional flow, simulating "arterial" and "venous" flow in tissues.

Bioreactors are reacting systems that involve transport of nutrients and oxygen from the perfusing medium to the cells, where metabolism produces waste materials and biological products that need to be transported back to the perfusing media for removal from the bioreactor. Depending upon bioreactor construction, transport in each compartment, and through the membranes separating compartments, is either by diffusion or combined convection and diffusion. The trend in BAL bioreactor design evolution has been to add more compartments with specific functions to simulate better the native organ. Indeed, [53] suggests that four-compartment bioreactors are necessary to enable integral oxygenation and distributed mass exchange with low gradients typical of the liver.

Hollow fiber membranes for nutrient perfusion have perm-selective properties that reject high molecular weight (HMW) molecules (>100–250 kDa, depending upon the membrane) and are used to isolate physically the perfusate from the cell compartment in order to mitigate the potential of either host (patient) vs graft (cells) rejection or graft vs host reaction and, in the case of porcine liver cells, prevent the transmission of xenogeneic disease such as porcine endogenous retrovirus. Use of perfusate hollow fiber membranes, however, introduces a resistance to desirable mass transport of nutrients and metabolites that needs to be considered in bioreactor design. Because oxygen transport to and consumption by liver cells has historically been considered to be a limiting feature in liver cell culture and maintenance, hollow fiber oxygenation membranes are used by the MELS CellModule and AMC-BAL to maintain local oxygen concentrations at a relatively constant level throughout the cell compartment.

Diffusion is often the main transport mechanism for low molecular weight (LMW) solutes, also in the presence of significant net transport of fluids across compartments (i.e., convection). Convection may significantly improve transport across compartments of HMW solutes (e.g., clotting proteins or growth factors), or protein-bound hydrophobic species, that may have important effects on cell behavior or the therapeutic BAL efficacy but whose diffusivity is much smaller than LMW solutes. The spatial profile of soluble nutrients and wastes, and the rate at which they are transferred across compartments, depends on the mass transport resistance of each compartment and their concentration in the compartment where they are supplied.

Each compartment, including membrane walls, in a BAL bioreactor can be described by the equations of motion coupled with mass transport (convective and diffusive) in a reacting system. The individual compartments are coupled through matching fluxes and species concentrations at the compartment boundaries. The

following sections present and discuss some of the advanced strategies proposed to enhance transport and liver cell metabolic activity in BAL bioreactors. Transport in the oxygenation membrane compartment, when present, is not discussed because resistance in this compartment is generally negligible.

2.1 Blood/Plasma Compartment

A still unresolved question in bioreactor design for extracorporeal BALs is whether perfusion by whole blood or plasma, continuously separated from the blood using a plasma separator such as a continuous centrifuge or plasmapheresis membrane module, is preferable [54]. In both cases, plasma is the carrier for soluble and protein-bound solutes into the bioreactor and liver-specific proteins and soluble factors such as clotting factors from the bioreactor. In whole blood, the red blood cells also act as efficient oxygen and carbon dioxide transporters. In both cases, in long term extracorporeal support, even with anticoagulant supplementation (e.g., heparin or citrate), proteins in the plasma (at least those of the complement cascade) may adhere to membrane surfaces in the plasma filter and/or the bioreactor, resulting in fouling and crippling of mass exchange and separation properties. Whole blood perfusion carries the additional risk that activation of the coagulation cascade may lead to platelet cell aggregation and blood clots that totally obstruct bioreactor perfusion.

Thus, bioreactor perfusion with either blood or plasma has both advantages and disadvantages. In the following, reference is made to plasma perfusion in the blood/plasma compartment. In fact, most proposed bioreactors process plasma that is continuously separated from the patient's blood by plasmapheresis or continuous centrifugation and that generally flows along the membrane length. Similar considerations apply to culture medium and blood, but for the higher capacity of blood to carry oxygen and the effects of the possible activation of the coagulation system. However, when comparing the clinical therapeutic efficacy of BALs it should be born in mind that, even though the same blood flow rate (e.g., 100–300 mL min^{-1}) is fed to the BALs, bioreactors based on a different technology actually treat a rather different fraction of the patient's plasma volume per unit time. In fact, in plasma treating bioreactors plasma is continuously removed from the blood in the extracorporeal loop and fed to the bioreactor at flow rates that do not generally exceed 20–60 mL min^{-1}, whereas bioreactors fed with whole blood treat a plasma flow rate about three times higher.

The resistance to solute transport from the bulk plasma to the membrane surface in reactors that use membranes to separate the cell compartment from the plasma compartment or from the bulk plasma to the cell construct surface in plasma per-fused bioreactors is generally lumped in a thin stagnant liquid film adjacent to the inner membrane surface or the cell construct. In the absence of significant net con-vective mass transport across the membrane or the construct surface, its actual value is estimated in terms of reciprocal mass transport coefficient k_c (i.e., the solute

conductivity) from nondimensional semi-empirical equations correlating the Sherwood number $Sh = k_c dD^{-1}$ with powers of nondimensional groups such as the Reynolds $Re = \rho U d\mu^{-1}$ and the Schmidt number $Sc = \mu r^{-1} D^{-1}$ (where ρ and μ are the plasma density and viscosity, respectively; U is the plasma velocity when the whole cross-sectional area is available for transport; d is the cell construct or membrane inner diameter; and D is the solute diffusivity in plasma) such as $Sh \propto Re^{\beta} Sc^{\gamma}$ [55]. The actual type of correlation and the value of the exponents depend on the channel and construct geometry. For flow in a nonporous cylindrical tube $\beta = \gamma = 1/3$ and Sh also depends on the 1/3 power of the membrane shape ratio d/L and the 0.14 power of the viscosity ratio at the wall and in the bulk. These correlations suggest that k_c increases with the β-th power of increasing plasma velocities and the reciprocal $(1 - \beta)$-th power of d.

The occurrence of secondary flows promoted by mechanical stirring (e.g., as in a Couette flat-sheet membrane module) or by the tortuous flow around cell constructs or aggregates, or obstacles in the flow channel (as in perfused cell bioreactors or in the MELS bioreactor when operated in perfusion mode, respectively) effectively mixes the plasma and causes k_c to increase with the liquid velocity more than when plasma flows in a cylindrical tube in laminar regime [56, 57]. For this reason, in recent years the rate at which plasma is circulated through the bioreactor in BALs has been kept fairly high, at values ranging from 50–400 to 100–300 mL min^{-1} for plasma and blood, respectively, depending on bioreactor geometry.

In membrane-compartmentalized cell bioreactors with a closed shell and equipped with permeable microfiltration membranes, operation at high linear plasma velocity results in increased axial pressure drops that enhance the occurrence of filtration-reabsorption flows (i.e., Starling flows) directed from the blood compartment towards the cell compartment at the bioreactor entrance and in the opposite direction at the exit. In fact, when the bioreactor shell is closed, higher pressure in the membrane lumen than in the shell at the bioreactor entrance drives convection of plasma across the membrane wall towards the cell compartment. As the pressure in the lumen drops along the membrane axis, it eventually becomes lower than that in the shell, and fluid is returned by convection to the lumen.

Brotherton and Chau [58] have nicely shown that Starling flows enhance mass transport towards and away from the cells to a significant extent only when cell density is low. This is the case when cells are seeded at low density in the bioreactor (as in the Arbios HepatAssist® BAL), or at the beginning of culture in bioreactors seeded with immortalized cell lines (as in the Vital Therapies ELAD® BAL). At cell densities approaching that of the liver (i.e., 10^8–10^9 cells mL^{-1}), the hydraulic resistance of the cell compartment is so high as to prevent significant Starling flows from occurring. Under these conditions, operation at high linear plasma velocity (i.e., high recirculation flow rates) reduces both the solute residence time in the blood compartment and the axial nutrient concentration gradient along the bioreactor length. However, even so, cells in the cell compartment may still be functioning under a diffusion-limited regime. In bioreactors that directly perfuse plasma through the cell compartment, such as the RAnD BAL and AMC-BAL, shear-sensitive liver cells are in direct contact with the plasma and are not protected by the membrane present in

the other hollow fiber based perfusion bioreactors. In this case, the maximal linear plasma velocity is limited by the shear forces that cells may tolerate without being damaged or torn away from the construct to which they adhere.

Optimization of transport and distribution of species, such as nutrients, in the plasma perfusion compartment, and the bioreactor as a whole, may have profound effects on cell behavior and the bioreactor performance. In most bioreactors proposed for BALs, plasma is generally assumed to distribute according to ideal plug flow patterns. Hence, plasma is assumed to be thoroughly mixed over sections perpendicular to the bioreactor length and solutes in any element of fluid entering the bioreactor are all assumed to have the same residence time. The determination of species residence time distribution (RTD) in a bioreactor by means of tracer experiments is a good statistical indicator of the actual flow pattern and mixing intensity in a given bioreactor [55]. The determination of the RTD is also an effective diagnostic tool for evidence of flow maldistribution caused by fluid channeling, the formation of stagnation regions in suboptimal bioreactor design, unexpected assembly problems, or presence of developing physical interactions in long-term operation.

Tracer experiments comparing a clinical-scale MELS CellModule, where liver cells are cultured in a 3D network between different semipermeable membranes, with a laboratory-scale flat bioreactor, where plasma or medium directly perfuses cells adherent to a collagen-coated flat substratum with oxygen delivery through oxygenation membranes placed above the cells in the plasma flow channel, have shown distinctly different RTDs for the tracer [59]. The experimental apparatus was optimized to minimize the dynamic response of the tubing and the solute sensing flow-through probes in order to challenge the bioreactor with a true stepwise changing tracer concentration in the entering stream. Under these conditions the bioreactor response could be analyzed in the time domain with decreased effects of experimental error in evaluation of the bioreactor RTD. In particular, in the MELS CellModule bioreactor operated in recycle, perfusion mode at high recycle ratios R (R being the recycle-to-feed flow rate) tracer RTD was comparable to that of an ideal continuous-flow stirred tank reactor (CSTR). Reducing R caused a significant reduction of the axial mixing intensity. In the flat bioreactor, operated in single-pass mode at low feed flow rate, the tracer RTD was similar to an ideal plug flow reactor (PFR). Operation at higher feed flow rate promoted significant axial dispersion and mixing, although not as effectively as in a CSTR. Real bioreactors, in particular large clinical-scale bioreactors, rarely follow ideal flow patterns unless their design and operation is carefully developed through combination of theoretical and experimental flow modeling.

Direct cell perfusion bioreactors, such as the AMC-BAL and RAnD BAL, where liver cells adhere to the fibers of nonwoven fabrics and form aggregates with possible bridging among neighboring cellular aggregates, have potential for flow maldistribution. This occurs when cells in some regions of the bioreactor form larger, more densely packed aggregates that feature a higher hydraulic resistance to plasma flow than other regions of the bioreactor. The result is that part of the plasma will channel preferentially through the regions of low hydraulic resistance where the nutrients come in contact with the cells for shorter-than-average times. In the

regions of high hydraulic resistance, plasma comes in contact with the cells for a longer-than-average time with the potential for rapid depletion of nutrients with concomitant starving of the cells (to death). In bioreactors equipped with oxygenation membranes another cause of flow maldistribution is nonuniform membrane distribution in the plasma perfusion compartment or physical interactions between neighboring membranes in long-term operation. The formation of a segregated low flow region in the middle of a flat bioreactor caused by oxygenation membranes sticking to one another after a few hours of operation is shown in Fig. 1a. The corresponding RTD for blue dextran, shown in Fig. 1b, exhibits two separate peaks resulting from the two segregated regions in the bioreactor.

Possible causes of flow maldistribution in membrane compartmentalized cell bioreactors include nonuniform diameter of the membranes used, the deformation or occlusion of membrane lumen caused by membrane potting or cutting with worn out blades, and the formation of blood clots when the bioreactor processes whole blood.

Independent of cause, flow maldistribution generally leads to lower-than-expected biotransformation yields and may cause unpredictable distributions of cellular activities and even local cell death.
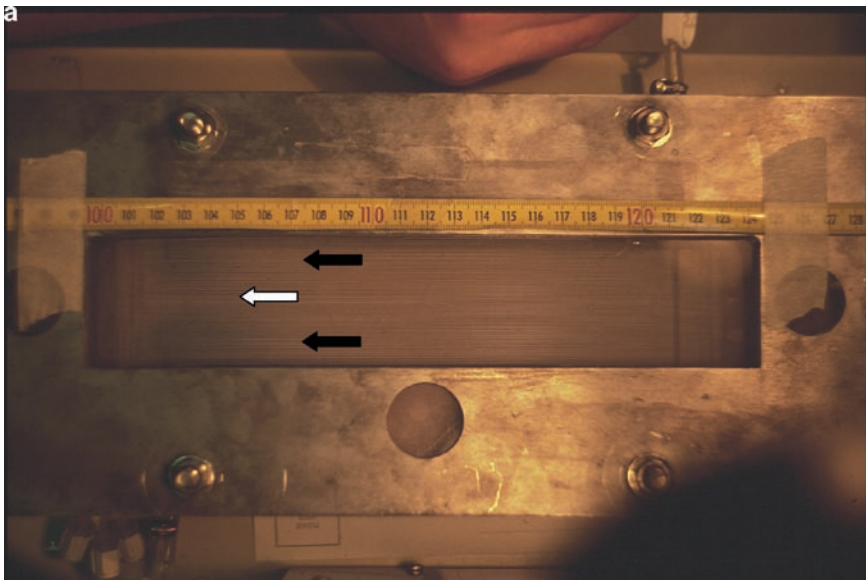

Fig. 1 a Photograph of tracing experiment where a flat bioreactor with oxygenation membranes hanging into the flow channel is subjected to a step challenge of blue dextran, after repeated testing for 5 h. The *black arrows* show the fast flow regions; the *white arrow* shows the low flow region in the middle of the flow channel caused by the oxygenation membranes sticking to one another. **b** RTD (*left axis, solid line*) and cumulative RTD, *F(t)*, (*right axis, open circles*) of a flat bioreactor with oxygenation membranes hanging into the flow channel after repeated testing for 5 h. The *black arrows* show the fast flow region; the *white arrow* shows the low flow region in the middle of the flow channel (see Fig. 1a) caused by the oxygenation membranes sticking to one another
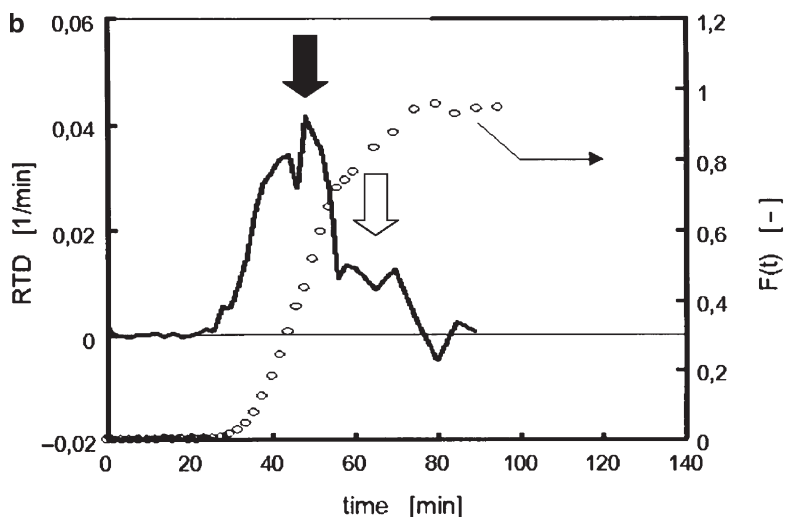
Fig. 1 (continued)

Optimization of bioreactor design and operation may minimize the detrimental effects of flow maldistribution and lead to a distribution of species that maximizes bioreactor performance and therapeutic efficacy (e.g., maintenance of cell viability and differentiated metabolic functions). In fact, in perfused cell bioreactors where species distribute in patterns similar to plug flow, metabolic products both form at rates monotonically increasing with increasing substrate concentrations and are produced by the cells more effectively than in completely mixed bioreactors (i.e., following a CSTR flow pattern). Thus, bioreactors that have plug flow characteristics require smaller cell mass for the production of a given mass of products per unit time. Likewise, larger amounts of intermediate metabolic products formed by series metabolic reactions would be produced than in completely mixed bioreactors [55].

Bioreactors featuring an established distribution of species may also be used to impose controlled gradients of oxygen, nutrients and growth factors over the cells to control their phenotype and resistance against blood-borne toxins. In fact, parenchymal liver tissue (i.e., hepatocytes) is characterized by variations of metabolic (e.g., carbohydrate metabolism) and detoxification (e.g., CYP450 enzymes) activities along the length of the sinusoid [60]. The effects of toxins and drugs have also regional specificity due to genetic and environmental cell differences [61]. This phenomenon is termed "liver zonation" and is thought to be regulated by gradients of oxygen and hormone concentrations, and extracellular matrix (ECM) composition [62, 63].

Allen and Bathia have shown that controlled steady state oxygen gradients may establish in a flat bioreactor where primary rat hepatocytes are cultured in adhesion on collagen-coated glass slides, by balancing the axial transport rate of dissolved oxygen and the cellular oxygen consumption rate (OCR) [24, 64]. They showed

that a validated transport-reaction model for the dissolved oxygen could be effectively used to adjust the bioreactor design and operation so as to establish near-physiological gradients of the dissolved oxygen concentration along the bioreactor length and avoid anoxic regions, at the same time.

In vivo, phosphoenolpyruvate carboxykinase (PEPCK) is expressed to a greater extent in periportal hepatocytes and CYP2B and CYP3A cytochrome P450 isoenzyme activities in perivenous cells. In vitro primary rat hepatocytes consistently expressed uniform PEPCK and CYP2B activities in the absence of an axial oxygen gradient. When exposed to a controlled continuous range of dissolved oxygen concentrations, the hepatocytes exhibited a heterogeneous distribution of PEPCK and CYP2B and CYP3A, when stimulated with glucagon, phenobarbital and dexamethasone respectively, mimicking their distributions in the natural liver tissue. In particular, cells in the bioreactor expressed higher PEPCK activities where they were exposed to higher dissolved oxygen concentrations, and higher CYP2B and CYP3A activities where they were exposed to lower dissolved oxygen concentrations.

Control of the axial dissolved oxygen concentration in the coculture of different liver cell types allows also for the exchange of paracrine signals among zonal subpopulations, as it occurs along the sinusoid in the natural liver. The powerful effect of physiological distributions of microenvironmental biochemical cues on CYP induction is testified by the dramatic increase reported in protein levels in the continuous-flow flat bioreactor as compared to standard Petri dish cultures challenged with the same 200 μM phenobarbital stimulus [64]. Cells exposed to a continuous range of dissolved oxygen concentrations did also respond differently when challenged with exogenous toxins similar to that happens in the natural liver [64].

Owing to the absence of red blood cells and the low solubility of O_2, the amount of O_2 carried by the plasma is much lower than that carried by the blood, and may be insufficient to meet the high OCR of hepatocytes, particularly hepatocytes attached to scaffolds [65] or exposed to toxins [66]. Supraphysiological dissolved oxygen tensions in the medium or plasma might help in meeting the hepatocyte OCR, but have been shown to compromise their viability possibly by the formation of free radicals [67, 68].

Solution to this problem was initially approached by positioning hollow fiber membranes originally developed for blood oxygenation in the plasma flow channel to replenish plasma with oxygen and remove carbon dioxide. In the first generation AMC BAL, microporous hydrophobic polypropylene hollow fiber membranes (ca. 0.6 m² area) were positioned as spacers in a spirally wound hydrophilic polyester nonwoven fabric, 0.4 mm thick, to supply oxygen locally [69]. In vitro tests showed evidence of anaerobic glycolytic metabolism in cells attached to the innermost regions of the fabric that was attributed to hypoxic culture conditions [70]. Recently, Mareels et al. [71] have reported on a model of momentum and oxygen transport in the space between neighboring fabric windings and into the fabric based on a commercial computational fluid dynamics (CFD) code. Simulations performed with this model confirmed that in the first generation bioreactor design only about 16–30% of the hepatocytes were adequately oxygenated. In their work, oxygenation

was considered adequate when cells could consume oxygen at 90% of their maximal uptake rate [72].

The important role of the internal membrane oxygen supply was confirmed by the fact that without internal oxygenation only a minor fraction of the cells (i.e., less then 6%) was predicted to be adequately oxygenated even when the dissolved oxygen tension in the medium entering the bioreactor was increased to ca. 300 mmHg. Model simulations also suggested that the distribution of the dissolved oxygen concentration could be enhanced by doubling the membrane packing density and/ or the oxygen content of the oxygenation gas. In vitro tests on small-scale bioreactors built according to the concept used for the AMC-BAL showed that primary porcine hepatocytes cultured in enhanced bioreactor designs in which the number of oxygena- tion membranes had been doubled and the thickness of the fabric more than halved (e.g., 0.183 mm vs 0.4 mm) exhibited slightly improved carbohydrate metabolism and functions over the standard design but differences generally were statistically insignificant [73]. Only when a 95% oxygen gas mixture was fed to the blood oxygena- tion membranes, resulting in a medium dissolved oxygen tension of ca. 250 mmHg, did the cells in the enhanced bioreactor design exhibit significantly reduced anaerobic glycolytic metabolism as compared to the standard design and only after ca. 6 days of culture. Correspondingly, cells eliminated ammonia and lidocaine, and produced urea and albumin at significantly higher rates.

Another way to increase the amount of oxygen carried by plasma (during treat- ment), or medium (in the stand-by phase), is to add a species that reversibly binds oxygen (i.e., an oxygen carrier), which can load large amounts of oxygen from an oxygen-rich gas source and release it to the cells, as hemoglobin does in the blood. Many biocompatible oxygen carriers have been proposed as blood substitutes. Solutions of cross-linked hemoglobin (Hb) have been proposed for their oxygen- carrying capacity and long half-life time. In fact, the cross-linking stabilizes the hemoglobin molecules and prevents the breakdown of the tetramer into the a1b1 and a2b2 dimers that are toxic to the kidneys [74]. However, even after cross-linking the hemoglobins may oxidize in hours and become toxic to cells in long-term cultures [75]. Risks of xenozoonosis should also not be ruled out when xenogeneic hemo- globin is used.

To alleviate the problems associated with the use of hemoglobin, Gordon and Palmer [76] have suggested supplementation of plasma or medium with intact bovine red blood cells (bRBCs), where Hb remains confined in the cells unless they undergo lysis. Supplementation of bovine red blood cells (at ca. 10% of the human hematocrit) to the medium used for the culture of C3A hepatoma cells in the ext- racapillary space of a membrane compartmentalized cell reactor was reported to establish a better oxygenated cell space than in the absence of the bRBCs for up to 16 days of culture. In fact, decreased lactate production-to-glucose consumption rate ratios and increased albumin synthesis were found when the bRBCs were added to the medium. However, when primary rat hepatocytes were cultured in the same bioreactor type, the supplementation of bRBCs to the medium (at ca. 2% of the human hematocrit) was not found to improve significantly cell oxygenation in the extracapillary space [77]. A transport-reaction model of the bioreactor suggests

that this was possibly due to the higher oxygen demand of primary cells and the reduced bRBC concentration used. In any case, bRBC settling in the reservoir tank, bRBC lysis, and the formation of metoxyhemoglobin were reported to be possible problems. The latter required bRBC replacement in the medium after a few days of culture [76].

Alternatively, hemoglobin encapsulation in polyethylene glycol-decorated phospholipids bilayers has been proposed to prevent its direct contact with cells or tissue [78]. Culture of human hepatoma HepG2 cells in the presence of liposome-encapsulated hemoglobin (LEH) was reported to be toxic and inhibit cell growth [79]. This was blamed on the cell capacity to take up lipoproteins and other lipids thus causing disruption of the microcapsule lipid bilayer, and the release of toxic free hemoglobin. A recent investigation shows that addition of 20% LEH by volume to the medium used for the short-term culture (i.e., 24 h) of primary rat hepatocytes adherent on collagen-coated flat substrata did not cause significant changes to cell morphology, nor to the rate of albumin synthesis. When cultured in a flat-plate perfused cell bioreactor without LEH, larger amounts of the same cells adherent on collagen-coated flat substrata gradually died towards the bioreactor outlet, as demonstrated by the morphological deterioration of their nuclei and cytoplasm. Supplementation of 20 vol.% of LEH to the medium prevented cell death along the bioreactor length and resulted in higher rates of albumin synthesis [78]. Prior to the use of LEH in BALs, their long-term toxicity should still be investigated for both primary adult hepatocytes and hepatocyte progenitor cells. In particular, the latter might take up lipids from the LEH wall and release free hemoglobin in their growth phase. Perfluorocarbon- (PFC) based oxygen carriers have also been proposed as blood substitutes. In fact, emulsions of one or more PFCs exhibit much higher solubility of oxygen and carbon dioxide than aqueous solutions. PFCs are synthetic very stable molecules (e.g., polytetrafluoroethylene, PTFE), chemically and biologically inert, which reversibly bind up to about 20 times more oxygen and carbon dioxide than water [80]. PFCs are immiscible with water, and have to be emulsified with surfactants to add them to plasma or culture medium with which they form an oil-in-water (o/w) type emulsion. PFC emulsions have been shown to increase oxygen transfer and cell proliferation of bacterial cultures [81]. Increased oxygen transfer and proliferation have also been reported for the culture of mouse hybridoma cells cultured in PFC o/w emulsions with average droplet diameter of 0.2 μm [82], and of rat kidney cells cultured at the interface between PFC and culture medium [83], respectively. Recently, addition to circulating plasma of 20% perfluorooctyl bromide (PFOB), emulsified with egg yolk lecithin and repeatedly treated by high-pressure homogenization to yield a narrow droplet diameter distribution of 0.2 mm mean value, has been proposed for BAL bioreactors [80]. In fact, PFOB has a low toxicity and is rapidly eliminated by the reticulo-endothelial system, if it enters the blood circulation. Egg yolk lecithine does not cause complement activation as other surfactants, such as the poloxamers (e.g., Pluronics®), do. In the proposed BAL design, the PFOB/plasma o/w emulsion is kept flowing continuously in the circulation loop where it is oxygenated in a membrane oxygenator and then flows through a radial flow bioreactor where porcine liver cells are cultured in adhesion to polyurethane

foam. The PFOB droplets are removed by ultrafiltration from the plasma emulsion leaving the bioreactor, and the plasma is returned to the patient at the same flow rate as the feed after mixing it with the concentrated blood leaving the plasma separation unit [84]. These emulsions were reported to be stable, could be easily sterilized, and could be maintained in the concentrated state by ultrafiltration without breaking them up. The presence of PFOB was reported not to have adverse effects on liver cells. However, addition of 20% PFOB to plasma did not change significantly the metabolic activity of liver cells adherent to polyurethane foams but for a higher rate of lidocaine clearance [85].

2.2   Cell Compartment

Because oxygen is an important nutrient that appears to modulate hepatocyte viability and function [62, 63] and is consumed at a high metabolic rate, researchers have focused considerable effort in understanding and enhancing oxygen transport throughout the cell mass in the cell compartment. The natural liver has an extensive sinusoidal network that maintains maximal diffusion distances from the blood to any cell in the liver at less than about 100 mm [86]. Because oxygen transport to the cell mass in a BAL bioreactor is also primarily by diffusion, by analogy, hypoxic regions may develop in the cell mass when the diffusion distance exceeds approximately 100 mm [26]. If so, diffusion distances place a severe limitation on the cell mass that can be supported by a single oxygen-providing source at cell concentrations nearing that in vivo and thus impact the scale-up of BAL bioreactors from laboratory scale to clinical scale.

One way of approaching this problem is to integrate an internal oxygenator into the cell compartment as in the MELS CellModule [7, 10]. The modular repeating unit of the CellModule bioreactor features a mat of oxygenation hollow fiber membranes interposed between two mats of plasma perfusion polyethersulphone hollow fiber membranes where oxygen-rich plasma or medium flows. Liver cells are cultured in the extracapillary space outside and among the membranes and receive oxygen from all the neighboring membranes – oxygenation as well as plasma perfusion. This design effectively reduces oxygen transport limitations and establishes physiological dissolved oxygen concentration gradients across the cell mass to an extent that depends on the oxygen partial pressure in the oxygenation gas flowing in the blood oxygenation membranes, the membrane packing density and the occurrence of plasma (or medium) filtrate perfusion across the cell mass. Consequently, CellModule bioreactors have been shown to support culture of porcine and human liver cells at in vivo concentrations [17, 36] and provide metabolic synthesis and detoxification activity [87].

Some BAL bioreactor designs use protein (e.g., type I collagen) or polysaccharide (e.g., alginate) gels to replace the natural ECM and provide the hepatocytes with three-dimensional scaffolding. Use of such matrix gels has been shown to enhance attachment and to promote polarization and differentiation of primary

hepatocytes. Drawbacks to the use of gels include lowered oxygen diffusivity relative to plasma or media and a likely increase in hydraulic resistance in the cell compartment that can hinder the occurrence of Starling flow [58]. Techniques have been proposed to enhance the oxygen transport capacity of ECM substitutes either by creating micropathways to induce some degree of convective oxygen transport or by adding oxygen carriers to the gel. A transport-enhanced ECM substitute was engineered by McClelland and Coger [88–90] that incorporated porous and hollow polystyrene microspheres (0.55 μm in diameter) into a collagen type I gel. The presence of the hydrophobic microspheres was shown by confocal microscopy to form a gap between the surface of each microsphere and the surrounding hydrophilic gel material [89] through which gaseous oxygen may be transported and may proceed through the pores of the microspheres. In fact, the gap thickness is estimated to be 10 Å larger than the 2.92 Å diameter of the oxygen molecule. The higher diffusivity of oxygen through the hollow microspheres than in the gel may also be expected to contribute the enhanced transport by augmenting the effective oxygen diffusivity in the transport-enhanced ECM substitute. In both cases, the extent of transport enhancement is expected to increase with the volumetric fraction of microspheres added to the gel.

The use of the transport-enhanced ECM substitute to entrap primary rat hepatocytes (at concentrations of the order of 10^6 cells mL^{-1}) was shown to increase the oxygen transport distance from the source from approximately 170 mm or less, in the absence of microspheres, to approximately 360 and 418 mm in the presence of 20 and 40 μL microspheres per mL of collagen solution, respectively [91]. Correspondingly, a larger fraction of the cells entrapped in the transport-enhanced ECM substitute farther from the oxygen source was viable and produced urea and albumin at higher specific metabolic rates than in a normal type I collagen gel [88]. Entrapment in the transport-enhanced ECM substitute was also shown to protect effectively the cells from exposure to hypoxia and hyperoxia [91].

Another way of approaching the problem of adequate oxygenation is to add an oxygenated PFC emulsion to a type I collagen gel [92]. A 60 wt% PFC emulsion, with an average 300 nm droplet diameter and stable for at least 75 days, was prepared by dissolving Perflubron (a commercially available PFC product) in an emulsion containing egg-yolk phospholipids, followed by ultrasonication. The resulting PFC-containing ECM substitute was prepared by mixing two parts type I collagen gelling solution with one part PFC emulsion on ice while bubbling with pure oxygen. Incubation at 37 °C for 30 min produced the final PFC-containing gel. The oxygen carrier included in the collagen gel is expected to increase the oxygen supply to adherent or embedded cells. Presumably, the carrier will initially release a bolus of the oxygen stored in the gel, which may be useful during cell attachment and, spreading when oxygen demand is highest. A long-term steady state follows where oxygen diffusion is believed to be enhanced by the presence of the oxygen carrier in the gel. Indeed, a culture of primary rat hepatocytes in adhesion on the PFC-containing gel was reported to have increased hepatocyte viability, cytochrome P450 activity, albumin secretion and urea production. More noticeably, rat hepatocytes embedded in the PFC-containing gel, and cultured in standard Petri dishes, secreted albumin

at rates that continuously increased over 8 days and that, at the end of culture, were approximately 350% and 166% higher than in adhesion culture on collagen in the absence and in the presence of serum, respectively. The long-term specific urea production rate of cells embedded in the PFC-containing gel was also approximately 76–79% higher than in adhesion on collagen. However, in all cases urea was produced at rates that continuously decreased in time with a residual 20–25% urea production rate after 8 days of culture. It is worth noticing that both oxygen transport enhancement techniques can be adapted to any BAL design where cells are embedded in a gel.

2.3 Membranes

All but one of the BAL bioreactors listed in Table 1 use perm-selective membranes to segregate the various compartments of the bioreactor. Their presence is seldom accounted for in the bioreactor design in spite of the fact that membrane volume accounts for approximately 15–20% of the bioreactor volume, based on the typical membrane diameter and wall thickness used for BALs, and that the mechanism of solute transport and its interactions with the membrane material may condition the bioreactor performance.

A primary purpose of the membrane separating the blood/plasma compartment from the cell compartment is immunologic: the membrane serves to isolate the cells from direct contact with the plasma in order to prevent both host-vs-graft and graft-vs-host reactions. Experience has demonstrated that ultrafiltration membranes that reject 90+% of solutes of molecular weight greater than about 70 kDa (i.e., membranes with a nominal molecular weight cut-off of about 70 kDa) and microfiltration membranes with maximal pore size of about 0.15 μm can effectively shield cells in the bioreactor from rejection. Such membranes also reduce the risk of xenozoonosis transmission (e.g., porcine endogenous retrovirus) to the patient when xenogeneic (porcine) cells are used [41, 93–95].

The transport and separation properties of membranes interposed between the plasma and the cell compartment influence and regulate the transport of water and soluble nutrients from the plasma to the cells and products and waste metabolites from the cells to the plasma.

Elegant analyses of convective-diffusive transport across such membranes have been presented [96–98] that provide the basis for understanding how the morphology of the membrane wall and membrane physical–chemical properties affect transport across the membranes. Initial BAL approaches used commercially available cellulose acetate dialysis membranes with low nominal molecular weight cut-off (hence, good barrier properties) and low hydraulic permeability that were approved by governmental agencies for use in medical treatments. Increasing awareness of the importance of membrane transport properties in bioreactor performance led to the use of membranes with as high a hydraulic permeability as possible provided that they exhibit the necessary separation properties to ensure protection of the cellular

graft. More recent versions of BAL bioreactors use highly permeable asymmetric ultrafiltration (i.e., hemo(dia)filtration) membranes with nominal molecular weight cut-off equal to or greater than about 100 kDa, e.g., polyethersulphone – MELS CellModule [35], and microfiltration (plasmapheresis) membranes with maximal pore size of about 0.2 mm, e.g., polysulphone – Arbios HepatAssist® BAL [94, 99]. At a given axial pressure drop, highly water permeable membranes are expected to provide higher Starling flows between the blood and the cell compartment with enhanced transport of mid-to-high MW nutrients and products towards and away from the cells. However, only bioreactors operating at low cell density would see improvements in Starling flow with more highly permeable membranes. At near in vivo cell densities, highly permeable membranes do not enhance transport across compartments to any significant extent.

Membrane composition is another important factor to consider in BAL bioreactor development. Many of the membranes used thus far consist of a hydrophobic polymeric backbone that is hydrophilized by chemical attachment of hydrophilic pendant moieties or by blending with hydrophilic polymers (as in the case of most commercial polysulphone membranes) or by physical treatment (as in the case of polypropylene membranes) [100]. Only a few BAL bioreactors use commercial membranes made of hydrophilic polymers (e.g., cellulose and its derivatives) with a nominal molecular weight cut-off of about 100 kDa.

Membranes themselves are but inert selective barriers, and soluble species with hydrophobic domains tend to adsorb on the hydrophobic polymeric backbone. Adsorption of mid-to-high MW proteins, greater than 5 kDa, and/or protein-bound solutes on the plasma/blood contacting membrane surface or on the pore surface into the membrane wall are not generally accounted for in transport models, but may significantly affect the bioreactor performance. In fact, in high-flux dialysis, hemofiltration or hemodiafiltration processes, adsorption of β2-microglobulin on polymethylmethacrylate or polyacrylonitrile membranes has been reported to increase significantly clearance of β2-microglobulin from the blood of uremic patients [101]. In similar fashion, membrane adsorption of hydrophobic hepatic toxins could transiently reduce the toxin concentration and exert a protective effect on the liver cells in the bioreactor. Adsorption of immune-competent proteins could also add to the membrane separation properties to protect the cellular graft from rejection. Adsorption has also been shown to have quantitative effects on lidocaine clearance in MELS CellModule-type bioreactors without cells in the cell compartment [87].

The downside of adsorption is that liver-specific protein products or growth factors might also be adsorbed on the membrane or be rejected by membranes whose pore size has been reduced by adsorption of mid-to-high MW proteins (a phenomenon termed fouling). In fact, the nominal molecular weight cut-off of polysulphone ultrafiltration membranes was shown to decrease significantly after contacting the blood for the adsorption of plasma proteins [102]. Protein adsorption on microfiltration symmetric membranes with a hydrophobic polymeric backbone was also shown to cause a dramatic reduction of the membrane water permeability [103]. Under these conditions, the actual concentration of growth factors in the cell

compartment may be much lower than in the blood compartment (and possibly be ineffective on cell behavior). Liver-specific products produced by the cells might also not be able to cross the membrane wall and reach the patient's blood circulation; accumulation in the cell compartment might possibly compromise the potential therapeutic efficacy of metabolically active cells.

Membranes separating the blood/plasma compartment from the cell compartment can also act as an attachment surface for attachment-dependent cells in the cell compartment. This has spurred research aimed at understanding the effect of membrane surface properties on liver cell metabolism. As nicely reviewed in Legallais et al. [28], several investigators have reported on the effect on liver cell adhesion and metabolism of the membrane polymeric material [104–108], surface wettability (i.e., hydrophilicity) [109], and roughness [110]. The reported results are qualitative and rather ambiguous. For instance, physically hydrophilized (i.e., wettable) polypropylene membranes appear to favor cell adhesion and metabolic activity [109], but Cuprophan® membranes made of highly hydrophilic regenerated cellulose were not reported to perform as well as membranes with a hydrophobic backbone [105]. This is possibly due to the fact that tests were generally performed in Petri dishes under time-varying and largely uncontrolled culture conditions. The ambiguous results may also be due the fact that many other chemical–physical surface properties known to affect cell behavior, such as the type and number of functional groups, the charge, the presence of crystalline regions, the surface roughness, among others, were varied (without real control) at the same time as membrane surface hydrophilicity.

Catapano et al. [111] proposed a technique to investigate the effect of surface wettability on liver cell metabolism by using membranes of a given polymer and surface roughness, physically modified to exhibit different amounts of oxygen at the surface, while minimizing the presence of different functional groups at the membrane surface. Liver cells were cultured in adhesion on membranes in a recycle bioreactor designed and operated to culture cells at steady, uniform and measurable concentrations of soluble species [109, 112]. Under these conditions, cells consistently expressed higher metabolic activities (e.g., cells consumed oxygen at higher rates) on more wettable membranes. Moreover, cells cultured on collagen were far more active than on uncoated membranes of similar wettability, possibly because of the presence of specific amino-acid sequences in the collagen. These preliminary results suggest that, when a significant fraction of cells are in direct contact with the membranes, the chemical–physical surface properties of the membrane may have quantitative effects on cell metabolic activities and on the transport of soluble metabolites in the bioreactor. In fact, when nutrients and oxygen supplied trough the membranes are consumed at a high rate by adherent cells on the membranes their concentration may be reduced so much as to starve the cells farther away from the membrane–blood interface. Thus, the advantages of using membranes with surface chemical–physical properties favoring cell metabolism (for the polymer of which they are made or because coated with natural protein substrata, such as collagen or Matrigel®) in clinical-scale bioreactors using 3D liver constructs might even be off-set by the increased diffusional nutrient limitations that they cause. In this respect, the quantitative

characterization of membrane effects on cell metabolic reactions could provide important information to optimize the bioreactor design and operation.

The presence of plasmapheresis (plasma filter) membranes is not generally accounted for in the evaluation of perfused cell bioreactors that operate with plasma. Such membranes are often used upstream from the bioreactor to continuously separate plasma for perfusion through the bioreactor from the blood stream. Because of significant, pressure-driven separation (microfiltration) of plasma from the blood, plasma filter membranes operate under more demanding conditions than membranes inside a bioreactor. As with membranes housed inside bioreactors, plasma filter membranes are subject to fouling not only from large molecular weight species but also from cellular deposition and clot formation. Accumulation of rejected or partially rejected large solutes at the membrane interface with the blood because of poor module design and operation may cause the permeate plasma flow rate to drop to levels unacceptable for therapeutic purposes. Fouling may also cause plasma proteins such as albumin to be largely retained in the blood stream, indirectly hindering the detoxification function of the BAL. While plasma filters can be replaced when performance drops below acceptable levels, the result is typically hemodilution in patients with already poor coagulation capacity – an undesirable clinical event.

3   Conclusions and Perspectives

Each of the proposed enhancement techniques presented in this chapter has been shown to bring about transport enhancements, though to different degrees, that yield better bioreactor metabolic performance in the short term. However, none of them has yielded stable cell expression of most metabolic functions typical of differentiated adult hepatocytes for longer than about a week. Nor has any technique been used for large clinical-scale BALs except for the internal oxygenation membranes in the MELS CellModule. Whether success from a single technique for enhancing transport in bioreactors on the scale of milliliters will yield similar transport enhancements when scaled to bioreactors hosting hundreds of grams of liver cells avidly consuming these nutrients remains to be seen. The integration of more transport enhancement techniques in the different compartments of a large scale bioreactor is more likely to result in more consistent transport and performance enhancements.

However, a large number of papers has been published in the last few years on the effects on hepatocyte metabolism of the characteristics of the scaffold to which they attach (i.e., geometry, morphology, physical–chemical properties, patterns of immobilized biochemical cues, etc.), and the coculture of different liver cells. Both are known to affect the liver cell organization and the hepatocyte phenotype. This suggests that techniques should be developed to control in vitro the microarchitecture of the liver cells after they are seeded into, or on, a scaffold to foster their organization in in vivo-like structures and promote mass transport mechanisms mimicking those of the liver *acinus*. The impact on cell behavior of controlling

the microenvironment and the mechanisms of mass transport has been demonstrated in mL-scale bioreactors, where the microfluidic environment allows the control of nanoliter fluid volumes and flows [113]. Integration of the knowledge of the mechanisms controlling cell arrangement and motility in porous scaffolds and of the factors affecting mass transport to, and away from, dense liver cell aggregates might provide design principles to better approximate the in vivo microenvironment also in clinical-scale bioreactors for BALs.

References

1. Bernal W, Auzinger, G, Sizer, E, and Wendon, J (2008) Intensive care management of acute liver failure. Sem Liver Dis 28:188–200
2. Lee WM, Squires RH Jr, Nyberg SL, Doo E, Hoofnagle JH (2008) Acute liver failure: summary of a workshop. Hepatology 47:1401–1415
3. Hadem J, Stiefel P, Bahr MJ, Tillmann HL, Rifai K, Klempnauer J, Wedemeyer H, Manns MP, Schneider AS (2008) Prognostic implications of lactate, bilirubin, and etiology in German patients with acute liver failure. Clin Gastroenterol Hepatol 6:339–345
4. Liou IW, Larson AM (2008) Role of liver transplantation in acute liver failure. Semin Liver Dis 28:201–209
5. Mullhaupt B, Dimitroulis D, Gerlach JT, Clavien PA (2008) Hot topics in liver transplantation: organ allocation – extended criteria donor – living donor liver transplantation. J Hepatol 48(Suppl 1):S58–S67
6. Polson J, Lee WM (2007) Etiologies of acute liver failure: location, location, location! [comment]. Liver Transplant 13:1362–1363
7. Gerlach JC, Zeilinger K, Patzer JF II (2008) Bioartificial liver: why, what, whither. Regen Med 3(4):575–595
8. Blei AT (2005) Selection for acute liver failure: have we got it right? Liver Transplant 11:S30–S34
9. Lin TY, Lee CS, Chen CC, Lian KY, Lin WSJ (1979) Regeneration of human liver after hepatic lobectomy studies by repeated liver scanning and repeated needle biopsy. Ann Surg 190:48–53
10. Gerlach J, Hout M, Gage K, Zeilinger K (2008) Liver cell-based therapy – bioreactors as enabling technology. In: Principles of regenerative medicine, Elsevier, Amsterdam, pp 1086–1105
11. Gruttadauria S, Mandala L, Miraglia R, Caruso S, Minervini MI, Biondo D, Volpes R, Vizzini G, Marsh JW, Luca Aet-al. (2007) Successful treatment of small-for-size syndrome in adult-to-adult living-related liver transplantation: single center series. Clin Transplant 21:761–766
12. Matsushita M, Nose Y (1986) Artificial liver. Artif Organs 10:378–384
13. Denis J, Opolon P, Delorme ML, Granger A, Darnis F (1979) Long-term extra-corporeal assistance by continuous haemofiltration during fulminant hepatic failure. Gastroenterol Clin et Biol 3:337–347
14. Knell AJ, Dukes DC (1976) Dialysis procedures in acute liver coma. Lancet 2:402–403
15. Mitzner SR, Stange J, Klammt S, Peszynski P, Schmidt R, Noldge-Schomburg G (2001) Extracorporeal detoxification using the molecular adsorbent recirculating system for critically ill patients with liver failure. J Am Soc Nephrol 12(Suppl 17):S75–S82
16. Opolon P (1979) High-permeability membrane hemodialysis and hemofiltration in acute hepatic coma: experimental and clinical results. Artif Organs 3:354–360
17. Sauer IM, Zeilinger K, Pless G, Kardassis D, Theruvath T, Pascher A, Goetz M, Neuhaus P, Gerlach JC (2003) Extracorporeal liver support based on primary human liver cells and albumin dialysis – treatment of a patient with primary graft non-function. J Hepatol 39:649–653

18. Willinger M, Schima H, Schmidt C, Huber L, Vogt G, Falkenhagen D, Losert U (1999) Microspheres based detoxification system: in vitro study and mathematical estimation of filter performance. Int J Artif Organs 22:573–582

19. Pascher A, Sauer IM, Hammer C, Gerlach JC, Neuhaus P (2002) Extracorporeal liver perfusion as hepatic assist in acute liver failure: a review of world experience [see comment]. Xenotransplantation 9:309–324

20. Gerlach JC, Zeilinger K (2002) Adult stem cell technology – prospects for cell based therapy in regenerative medicine. Int J Artif Organs 25:83–90

21. Gerlach JC, Kloppel K, Muller C, Schnoy N, Smith MD, Neuhaus P (1993) Hepatocyte aggregate culture technique for bioreactors in hybrid liver support systems. Int J Artif Organs 16:843–846

22. Nyberg SL, Shatford RA, Hu WS, Payne WD, Cerra FB (1992) Hepatocyte culture systems for artificial liver support: implications for critical care medicine (bioartificial liver support) Crit Care Med 20:1157–1168

23. Dahmen U, Madrahimov N, Madrahimova F, Ji Y, Schenk A, Dirsch O (2008) Small-for-Size syndrome in the rat: does size or technique matter? J Surg Res 149(1):15–26

24. Allen JW, Hassanein T, Bhatia SN (2001) Advances in bioartificial liver devices. Hepatology 34:447–455

25. Busse B, Smith MD, Gerlach JC (1999) Treatment of acute liver failure: hybrid liver support. A critical overview. Langenbecks Arch Surg 384:588–599

26. Catapano G, De Bartolo L, Lombardi CP, Drioli E (1996) The effect of oxygen transport resistances on the viability and functions of isolated rat hepatocytes. Int J Artif Organs 19:61–71

27. Catapano G, Gerlach JC (2007) Bioreactors for liver tissue engineering. In: Ashammakhi N , Reis R , Chiellini E (eds.) Topics in tissue engineering, pp 1 – 41

28. Legallais C, David B, Dore E (2001) Bioartificial livers (BAL): current technological aspects and future developments. J Membr Sci 181:81–95

29. Park JK, Lee DH (2005) Bioartificial liver systems: current status and future perspective. J Biosci Bioeng 99:311–319

30. Patzer JFII (2001) Advances in bioartificial liver assist devices. Ann N Y Acad Sci 944:320–333

31. Mazariegos GV, Kramer DJ, Lopez RC, Shakil AO, Rosenbloom AJ, DeVera M, Giraldo M, Grogan TA, Zhu Y, Fulmer ML et-al. (2001) Safety observations in phase I clinical evaluation of the excorp medical bioartificial liver support system after the first four patients. ASAIO J 47:471–475

32. Mazariegos GV, Patzer JF, Lopez RC, Giraldo M, deVera ME, Grogan TA, Zhu Y, Fulmer ML, Amiot BP, Kramer DJ (2002) First clinical use of a novel bioartificial liver support system (BLSS) Am J Transplant 2:260–266

33. Millis JM, Cronin DC, Johnson R, Conjeevaram H, Conlin C, Trevino S, Maguire P (2002) Initial experience with the modified extracorporeal liver-assist device for patients with fulminant hepatic failure: system modifications and clinical impact. Transplantation 74:1735–1746

34. Demetriou AA, Brown RS, Busuttil RW, Fair J, McGuire BM, Rosenthal P, Esch JSA, Lerut J, Nyberg SL, Salizzoni M et-al. (2004) Prospective, randomized, multicenter, controlled trial of a bioartificial liver in treating acute liver failure. Ann Surg 239:660–667

35. Gerlach JC, Mutig K, Sauer IM, Schrade P, Efimova E, Mieder T, Naumann G, Grunwald A, Pless G, Mas A et al. (2003) Use of primary human liver cells originating from discarded grafts in a bioreactor for liver support therapy and the prospects of culturing adult liver stem cells in bioreactors: a morphologic study. Transplantation 76:781–786

36. Sauer IM, Kardassis D, Zeillinger K, Pascher A, Gruenwald A, Pless G, Irgang M, Kraemer M, Puhl G, Frank J et al. (2003) Clinical extracorporeal hybrid liver support – phase I study with primary porcine liver cells. Xenotransplantation 10:460–469

37. van de Kerkhove MP, Di Florio E, Scuderi V, Mancini A, Belli A, Bracco A, Dauri M, Tisone G, Di Nicuolo G, Amoroso P et al. (2002) Phase I clinical trial with the AMC-bioartificial liver. Int J Artif Organs 25:950–959

38. Morsiani E, Pazzi P, Puviani AC, Brogli M, Valieri L, Gorini P, Scoletta P, Marangoni E, Ragazzi R, Azzena G et-al. (2002) Early experiences with a porcine hepatocyte-based bioartificial liver in acute hepatic failure patients. Int J Artif Organs 25:192–202

39. Baquerizo A, Mhoyan A, Shirwan H, Swensson J, Busuttil RW, Demetriou AA, Cramer DV (1997) Xenoantibody response of patients with severe acute liver failure exposed to porcine antigens following treatment with a bioartificial liver. Transplant Proc 29:964–965

40. Ellis AJ, Hughes RD, Wendon JA, Dunne J, Langley PG, Kelly JH, Gislason GT, Sussman NL, Williams R (1996) Pilot-controlled trial of the extracorporeal liver assist device in acute liver failure. Hepatology 24:1446–1451

41. Kuddus R, Patzer JF II, Lopez R, Mazariegos GV, Meighen B, Kramer DJ, Rao AS (2002) Clinical and laboratory evaluation of the safety of a bioartificial liver assist device for potential transmission of porcine endogenous retrovirus. Transplantation 73:420–429

42. Millis JM, Cronin DC, Johnson R, Conjeevaram H, Brady L, Trevino S, Conlin C, Brotherton J, Traglia D, Dane G et-al. (2001) Safety of continuous human liver support. Transplant Proc 33:1954

43. Pitkin Z, Mullon C (1999) Evidence of absence of porcine endogenous retrovirus (PERV) infection in patients treated with a bioartificial liver support system. Artif Organs 23:829–833

44. Kamohara Y, Rozga J, Demetriou AA (1998) Artificial liver: review and Cedars-Sinai experience. J Hepato-Biliary-Pancreatic Surg 5:273–285

45. Balis UJ, Behnia K, Dwarakanath B, Bhatia SN, Sullivan SJ, Yarmush ML, Toner M (1999) Oxygen consumption characteristics of porcine hepatocytes. Metab Eng 1:49–62

46. Catapano G, De Bartolo L, Lombardi CP, Drioli E (1996) The effect of catabolite concentration on the viability and functions of isolated rat hepatocytes. Int J Artif Organs 19:245–250

47. Freshney R (2000) Culture of animal cells – a manual of basic techniques, 4th edn. Wiley, New York

48. Rotem A, Toner M, Bhatia S, Foy BD, Tompkins RG, Yarmush ML (1994) Oxygen is a factor determining in-vitro tissue assembly – effects on attachment and spreading of hepatocytes. Biotechnol Bioeng 43:654–660

49. Mueller-Klieser WF, Sutherland RM (1982) Oxygen tensions in multicell spheroids of two cell lines. Br J Cancer 45:256–264

50. Sutherland RM, Sordat B, Bamat J, Gabbert H, Bourrat B, Mueller-Klieser W (1986) Oxygenation and differentiation in multicellular spheroids of human colon carcinoma. Cancer Res 46:5320–5329

51. Tannock IF (1972) Oxygen diffusion and the distribution of cellular radiosensitivity in tumours. Br J Radiol 45:515–524

52. Vaupel P (1977) Hypoxia in neoplastic tissue. Microvasc Res 13:399–408

53. Xu ASL, Luntz TL, Macdonald JM, Kubota H, Hsu E, London RE, Reid LM (1999) Lineage and biology and liver. In: Lanza RP, Langer R, Vacanti J (eds.) Principles of tissue engineering, 2nd edn. San Diego, Academic Press, chap. 41, pp 559–598

54. Patzer JF II, Campbell B, Miller R (2002) Plasma versus whole blood perfusion in a bioartificial liver assist device. ASAIO J 48:226–233

55. Levenspiel O (1999) Chemical Reaction Engineering, 3rd edn. Wiley, New York

56. Catapano G, Papenfuss HD, Wodetzki A, Baurmeister U (2001) Mass and momentum transport in extra-luminal flow (ELF) membrane devices for blood oxygenation. J Membr Sci 184:123–135

57. Ozisik NM (1985) Heat Transfer. McGraw-Hill, New York

58. Brotherton JD, Chau PC (1996) Modeling of axial-flow hollow fiber cell culture bioreactors. Biotechnol Prog 12:575–590

59. Catapano G, Euler M, Gaylor JDS, Gerlach J (2001) Characterization of the distribution of matter in hybrid liver support devices where cells are cultured in a 3D membrane network or on flat substrata. Int J Artif Organs 24:102–109

60. Jungermann K, Thurman RG (1992) Hepatocyte heterogeneity in the metabolism of carbohydrates. Enzyme 46:33–58

61. Lindros KO (1997) Zonation of cytochrome P450 expression, drug metabolism and toxicity in liver. General Pharmacol 28:191–196
62. Jungermann K, Kietzmann T (2000) Oxygen: modulator of metabolic zonation and disease of the liver. Hepatology 31:255–260
63. Kietzmann T, Jungermann K (1997) Modulation by oxygen of zonal gene expression in liver studied in primary rat hepatocyte cultures. Cell Biol Toxicol 13:243–255
64. Allen JW, Khetani SR, Bhatia SN (2005) In vitro zonation and toxicity in a hepatocyte bioreactor. Toxicol Sci 84:110–119
65. Foy BD, Rotem A, Toner M, Tompkins RG, Yarmush ML (1994) A device to measure the oxygen uptake rate of attached cells: importance in bioartificial organ design. Cell Transplant 3:515–527
66. Catapano G, De Bartolo L (2002) Combined effect of oxygen and ammonia on the kinetics of ammonia elimination and oxygen consumption of adherent rat liver cells. Int J Artif Organs 25(2):151–157
67. Fariss MW (1990) Oxygen toxicity: unique cytoprotective properties of vitamin E succinate in hepatocytes. Free Radical Biol Med 9:333–343
68. Martin H, Sarsat JP, Lerche-Langrand C, Housset C, Balladur P, Toutain H, Albaladejo V (2002) Morphological and biochemical integrity of human liver slices in long-term culture: effects of oxygen tension. Cell Biol Toxicol 18:73–85
69. Flendrig LM, la Soe JW, Jorning GG, Steenbeek A, Karlsen OT, Bovee WM, Ladiges NC, te Velde AA, Chamuleau RA (1997) In vitro evaluation of a novel bioreactor based on an integral oxygenator and a spirally wound nonwoven polyester matrix for hepatocyte culture as small aggregates. J Hepatol 26:1379–1392
70. van de Kerkhove MP, Poyck PP, van Wijk AC, Galavotti D, Hoekstra R, van Gulik TM, Chamuleau RA (2005) Assessment and improvement of liver specific function of the AMC-bioartificial liver. Int J Artif Organs 28:617–630
71. Mareels G, Poyck PP, Eloot S, Chamuleau RA, Verdonck PR (2006) Three-dimensional numerical modeling and computational fluid dynamics simulations to analyze and improve oxygen availability in the AMC bioartificial liver. Ann Biomed Eng 34:1729–1744
72. Patzer JF II (2004) Oxygen consumption in a hollow fiber bioartificial liver--revisited. Artif Organs 28:83–98
73. Poyck PP, Mareels G, Hoekstra R, van Wijk AC, van der Hoeven TV, van Gulik TM, Verdonck PR, Chamuleau RA (2008) Enhanced oxygen availability improves liver-specific functions of the AMC bioartificial liver. Artif Organs 32:116–126
74. Chang TM (1999) Future prospects for artificial blood. Trends Biotechnol 17:61–67
75. Alayash AI (1999) Hemoglobin-based blood substitutes: oxygen carriers, pressor agents, or oxidants? Nat Biotechnol 17:545–549
76. Gordon J, Palmer AF (2005) Impact of increased oxygen delivery via bovine red blood cell supplementation of culturing media on select metabolic and synthetic functions of C3A hepatocytes maintained within a hollow fiber bioreactor. Artif Cells Blood Subst Biotechnol 33(3):297–306
77. Sullivan JP, Palmer AF (2006) Targeted oxygen delivery within hepatic hollow fiber bioreactors via supplementation of hemoglobin-based oxygen carriers. Biotechnol Prog 22:1374–1387
78. Naruto H, Huang H, Nishikawa M, Kojima N, Mizuno A, Ohta K, Sakai Y (2007) Feasibility of direct oxygenation of primary-cultured rat hepatocytes using polyethylene glycol-decorated liposome-encapsulated hemoglobin (LEH) J Biosci Bioeng 104:343–346
79. Sakai Y, Huang H, Naruto H, Nishikawa M, Kojima N, Mizuno A, Ohta K (2006) Use of liposome-encapsulated hemoglobin (LEH) as an oxygen carrier to cultured cells. In: Peppas NA, Hoffman AS, Kanamori T, Tojo K (eds.) Advances in medical engineering, drug delivery systems and therapeutic systems, American Institute of Chemical Engineering, New York, pp 45–50
80. Moolman FS (2004) Oxygen carriers for a novel bio-artificial liver support system. Pretoria, University of Pretoria

81. Lowe KC, Anthony P, Wardrop J, Davey MR, Power JB (1997) Perfluorochemicals and cell biotechnology. Artif. Cells Blood Substitutes Immobilization Biotechnol 25:261–274
82. Ju LK, Armiger WB (1992) Use of perfluorocarbon emulsions in cell culture. Biotechniques 12:258–263
83. Sanfilippo B, Ciardiello F, Salomon DS, Kidwell WR (1988) Growth of cells on a perfluoro-ocarbon-medium interphase: a quantitative assay for anchorage-independent cell growth. In Vitro Cell Dev Biol 24:71–78
84. Van Der Merwe S, Moolman FS, Bond RP, Van Wyk AJ (2002) Bio-reactor device, PCT, Patent WO02/22775
85. Nieuwoudt MJ, Moolman SF, Van Wyk KJ, Kreft E, Olivier B, Laurens JB, Stegman FG, Vosloo J, Bond R, van der Merwe SW (2005) Hepatocyte function in a radial-flow bioreactor using a perfluorocarbon oxygen carrier. Artif Organs 29:915–918
86. Koeppen BM, Stanton BA (2008) Berne and Levy Physiology, 6th edn. Mosby, St. Louis, MO
87. Unger JK, Catapano G, Horn NA, Schroers A, Gerlach JC, Rossaint R (2000) Comparative analysis of metabolism of medium- and plasma perfused primary pig hepatocytes cultured around a 3D membrane network. Int J Artif Organs 23:104–110
88. McClelland RE, Coger RN (2000) Use of micropathways to improve oxygen transport in a hepatic system. J Biomechan Eng 122:268–273
89. McClelland RE, Coger RN (2004) Effects of enhanced O(2) transport on hepatocytes packed within a bioartificial liver device. Tissue Eng 10:253–266
90. McClelland RE, MacDonald JM, Coger RN (2003) Modeling O2 transport within engineered hepatic devices. Biotechnol Bioeng 82:12–27
91. Niu M, Clemens MG, Coger RN (2008) Optimizing normoxic conditions in liver devices using enhanced gel matrices. Biotechnol Bioeng 99:1502–1512
92. Nahmias Y, Kramvis Y, Barbe L, Casali M, Berthiaume F, Yarmush ML (2006) A novel formulation of oxygen-carrying matrix enhances liver-specific function of cultured hepato-cytes. FASEB J 20:2531–2533
93. Gleissner M, Bornemann R, Stemerowicz R, Meissler M, Neuhaus P, Gerlach JC (1997) Immunoisolation of hybrid liver support systems by semipermeable membranes. Int J Artif Organs 20:644–649
94. Mullon C (1999) Bioartificial organs may help reduce risk of zoonosis in xenotransplantation. Artif Organs 23:366–367
95. Nyberg SL, Hibbs JR, Hardin JA, Germer JJ, Persing DH (1999) Transfer of porcine endog-enous retrovirus across hollow fiber membranes: significance to a bioartificial liver [see comment]. Transplantation 67:1251–1255
96. Baker RW (2004) Membrane transport theory. In: Membrane technology and applications, 2nd edn. New York, Wiley, chap 2, 15–84
97. Michaels AS (1966) Operating parameters and performance criteria for hemodialyzers and other membrane-separation devices. Trans Am Soc Artif Intern Organs 12:387–392
98. Nakao S, Kinura S (1982) Models of membrane transport phenomena and their applications for ultrafiltration data. J Chem Eng Jpn 15:200
99. Watanabe FD, Mullon CJ, Hewitt WR, Arkadopoulos N, Kahaku E, Eguchi S, Khalili T, Arnaout W, Shackleton CR, Rozga J et-al. (1997) Clinical experience with a bioartificial liver in the treatment of severe liver failure. A phase I clinical trial. Ann Surg 225:484–491; discussion 491–484
100. Catapano G, Vienken J (2008) Medical applications. In: Li N , Fane AG, Ho WSW, Matsuura T (eds.) Advanced membrane science and technology, Wiley, New York
101. Clark WR, Hamburger RJ, Lysaght MJ (1999) Effect of membrane composition and structure on solute removal and biocompatibility in hemodialysis. Kidney Int 56:2005–2015
102. Feldhoff P, Turnham T, Klein E (1984) Effect of plasma proteins on the sieving spectra of hemofilters. Artif Organs 8:186–192
103. Jonsson G (1986) Transport phenomena in ultrafiltration: membrane selectivity and boundary layer phenomena. Pure Appl Chem 58:1647–1656

104. Gerlach J, Stoll P, Schnoy N, Bucherl ES (1990) Membranes as substrates for hepatocyte adhesion in liver support bioreactors. Int J Artif Organs 13:436–441
105. Gerlach JC, Schnoy N, Vienken J, Smith M, Neuhaus P (1996) Comparison of hollow fibre membranes for hepatocyte immobilisation in bioreactors. Int J Artif Organs 19:610–616
106. Jozwiak A, Karlik W, Wiechetek M, Werynski A (1998) Attachment and metabolic activity of hepatocytes cultivated on selected polymeric membranes. Int J Artif Organs 21:460–466
107. Krasteva N, Seifert B, Hopp M, Malsch G, Albrecht W, Altankov G, Groth T (2005) Membranes for biohybrid liver support: the behaviour of C3A hepatoblastoma cells is dependent on the composition of acrylonitrile copolymers. J Biomater Sci Polym Ed 16:1–22
108. Qiang S, Yaoting Y, Hongyin L, Klinkmann H (1997) Comparative evaluation of different membranes for the construction of an artificial liver support system. Int J Artif Organs 20:119–124
109. Catapano G, Di Lorenzo MC, Della Volpe C, De Bartolo L, Migliaresi C (1996) Polymeric membranes for hybrid liver support devices: the effect of membrane surface wettability on hepatocyte viability and functions. J Biomater Sci Polym Ed 7:1017–1027
110. De Bartolo L, Catapano G, Della Volpe C, Drioli E (1999) The effect of surface roughness of microporous membranes on the kinetics of oxygen consumption and ammonia elimination by adherent hepatocytes. J Biomater Sci Polym Ed 10:641–655
111. Catapano G, Speranza G, Maniglio D, De Bartolo L, Della Volpe C (2002) Bioreactor type and operating conditions influence cell response to polymeric material properties. Proceedings of the IEEE-EMBS Special Topic Conference on "Molecular, Cellular and Tissue Engineering", Genova (Italy), 6–9 June 2002
112. Catapano G, De Bartolo L (1998) Technique for the kinetic characterization of the metabolic reactions of hepatocytes in adhesion culture. Biotechnol Prog 14:500–507
113. Walker GM, Zeringue HC, Beebe DJ (2004) Microenvironment design considerations for cellular scale studies. Lab on a Chip 4:91–97

Sensors in Disposable Bioreactors Status and Trends

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Abstract For better control of productivity and product quality, detailed monitoring of various parameters is required. Since disposable bioreactors become more and more important for biotechnological applications, adequate sensors for this type of reactor are necessary. The required properties of sensors used in disposable reactors differ from those of sensors for multiuse reactors. For example, sensors which are in direct contact with the medium must be inexpensive, but do not need a long life-time, since they can be used only once.

This chapter gives an overview on the state of the art and future trends in the field of sensors suited for use in disposable bioreactors. The main focus here is on in situ sensors, which can be based on optical, semiconductor and ultrasonic technologies, but current concepts for disposable sampling units are also reviewed.

Keywords Disposable reactors • Disposable sensors • Electrochemical sensors • In situ microscopy • Optical sensing • Ultrasound

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Abbreviations

κ	Adiabatic compressibility
κ_c	Conductivity
κ_{cell}	Cell constant
ρ	Density
ATR	Attenuated total reflectance
CA	Commercially available
CIP	Cleaning in place
c_{US}	Velocity of ultrasonic wave
I_{Drain}	Drain current
IR	In research
ISFET	Ion-sensitive field-effect transistor
JFET	Junction field-effect transistor
MOSFET	Metal oxide semiconductor field-effect transistor
NA	Not available
NIR	Near infrared
PAT	Process analysis technology
pHFET	pH-sensitive ISFET
R	Resistance
SIP	Sterilization in place
SWIR	Short wave infrared
U_{Drain}	Drain voltage
U_{Gate}	Gate voltage

1 Introduction

Bioprocess analysis is required to monitor bioprocess parameters in detail to control productivity and product quality better. Its importance has grown significantly during the last 2 years due to the PAT (process analysis technology) initiative of the FDA, which supports process analytical techniques for development, production, and quality

management, particularly in the food and pharmaceutical industries. In this area there is a growing interest in disposable bioreactors and thus adequate disposable sensors. Since these systems can be obtained presterilized and do not require cleaning after use, the development and optimization of new processes is achieved in a much shorter time than with traditional nondisposable systems. This is particularly important in drug development where the time from development to market is crucial for product success. In addition, the investment capital is low compared to that required for traditional systems, while consumable costs are higher. This leads to a more balanced cost distribution over time, resulting in an increased flexibility during the development process [1, 2].

Biotechnological processes generally require the monitoring of physical (e.g., temperature, conductivity), chemical (e.g., glucose, pH, pO_2, pCO_2) and biological (e.g., cell density and viability) parameters. The sensor selectivity, sensitivity, response, and analysis time must be matched to the corresponding bioprocess. For example, fast-growing organisms such as fungi and bacteria require analysis frequencies in the region of minutes, while mammalian and plant cell cultures only require hourly monitoring due to their slower growth rates.

Sensors can be coupled to a bioreactor via in situ, online and ex situ configurations. The important distinction among these options is whether the medium is monitored directly (in situ sensors), moved to a special part of the bioreactor (online sensors), or removed from the bioreactor (ex situ sensors). Online sensors are positioned in a bypass, for example to remove bubbles that might interfere with the measurement. Both in situ and online sensors deliver a continuous stream of information and have very short response times. In situ and online sensors may also be noninvasive or in direct contact with the cultivation medium. Sensors in direct contact with the medium must be sterilizable (autoclavable) and thus sensors such as biosensors cannot be used since the biological component is destroyed during the sterilization process. Up to now, the in situ and online sensors most commonly used in biotechnology are based on electrochemical principles (pH, pO_2, conductivity, temperature). Ex situ sensors are not directly connected to a bioreactor and require a sterile sampling unit. This is highly challenging as the delay time between sampling and analysis must be short to enable process regulation. For further details we refer to numerous review articles published on bioprocess analysis [3–10].

Sensor systems for disposable bioreactors must be cost effective (on a per use basis) and reliable. If the sensor itself is disposable (for in situ or online application) it must be inexpensive but need not have a long lifetime. Thus, traditional electrodes, due to their high price, cannot be used as disposable sensors. Instead, several approaches can be used to meet these requirements. For example, inexpensive sensing elements can be located inside a disposable reactor and used with reusable analytical equipment outside the reactor. Inexpensive, single-use sensors can also be based on semiconductor devices (e.g., pH-ISFETs) and placed either in the gas phase (headspace, inlet, outlet) or into the cultivation broth for liquid-phase analysis (temperature, pH, pO_2). Another approach is to use optical sensors, with which monitoring can be performed noninvasively through a transparent window. Nondisposable sensors can be interfaced with the bioreactor in an ex situ manner. Commonly used

disposable bioreactors contain modules where manual sampling can be performed or disposable sampling systems can be connected to the bioreactor. However, disposable sampling systems for continuous sterile sampling are not yet on the market.

In this chapter an overview of the state of the art and future trends in the field of sensors suited for use in disposable bioreactors is given. Specific ex situ sensor systems (e.g., HPLC) are not further considered in this chapter; instead, current concepts for disposable sampling units are reviewed. The main focus here is on disposable in situ sensors, which can be based on optical, semiconductor, and ultrasonic technologies. Examples are given for measured variables that are particularly important in biotechnology, including temperature, conductivity, pH, pO_2, pCO_2, important metabolites (glucose, ethanol, lactate), and cell density/biomass.

2 Disposable Sampling Systems for Ex Situ Analysis

Continuous sampling from a bioreactor is required for the use of ex situ sensors. This is a significant challenge as the sterility of the cultivation must be retained, while the dead volume of the sampling device needs to be small. Furthermore, the analysis frequency should be as high as possible to enable process control. Sampling from a bioreactor is generally highly invasive. In addition to the risk of contamination, sampling results in an increased sheer stress for the cells and leads to a change in the composition of the cultivation broth, particularly when cell-free samples are taken [11].

The simplest means to accomplish cell-free sampling is by using a tube with a sterile filter connected to a peristaltic pump. Such systems are inexpensive and can easily be made to be disposable, but their major drawback is a large dead volume. This problem can be overcome by filtration probes that use a microfiltration membrane as sterile barrier and are positioned inside the bioreactor [12]. These devices, such as the ESIP probe by Trace Biotech AG (Braunschweig, Germany), are usually designed for repeated uses in a steel reactor. Although a semidisposable system, in which the filtration membrane is replaced after every cultivation run, was recently developed by Groton Biosystems (Boxborough, Massachusetts, USA), these systems are not yet available for use in fully disposable reactors [13].

Removing cell-containing samples from a bioreactor is generally more difficult because the sampling system is in direct contact with the cell suspension, which may compromise sterility. It is also necessary to stop all metabolic activities in the sample so that the sample reflects the composition inside the bioreactor. This can be done by freezing or the addition of inactivation agents but further increases the system complexity.

To avoid these problems, several innovative disposable sampling systems for cell-containing samples have been developed. Some are based on thermoplastic tubing that can be used to perform aseptic welds and can be sealed simply by heating; these allow one to acquire samples from closed systems like a bag bioreactor. This technique was first applied with small diameter tubing by Terumo Medical Corporation

and later used in systems with larger diameters and higher flow rates by several companies including Wave Biotech AG (Tagelswangen, Suisse) and Sartorius-Stedim AG (Göttingen, Germany). In the sampling process, a presterilized sampling container, including a needleless syringe, can be welded to the sampling module of the bag bioreactor. A sample is then pumped into the container and the connection can be severed afterward by heat-sealing. This enables easy sampling without the risk of contamination and with no need for a Grade A clean zone. However, only a few samples can be removed over a cultivation process [14].

Other fully disposable sampling systems that can be applied in disposable and classical stainless steel bioreactors are distributed by Cellexus Biosystems (Cambridgeshire, UK) and Millipore (Billerica, Massachusetts, USA). The Cellexus system is connected to a bioreactor by a presterilized Luer connection including a one-way valve to prevent the sample from flowing back into the reactor. The sample is withdrawn from the reactor by a syringe and directed through a sample line into a reservoir. Connected to the sample line are up to six sealed sample pouches, the seal of which can be opened by flexing. The sample from the reservoir can then be pushed into the pouches that are subsequently severed by a mechanical sealer, resulting in sealed, sterile samples [15].

The patented Millipore system consists of a port insert that can be fitted to several reactor sideports and a number of flexible conduits that can be opened and closed individually for sampling and are connected to flexible, disposable sampling containers. During the sampling process, one conduit is opened and the sample can flow into the sampling container, which may be disconnected by heat sealing. Samples in the range of 5–1,000 mL may be removed, but the maximum number of samples is limited by the number of available conduits in each sampling module [16].

All of these disposable sampling systems enable aseptic sampling from several different bioreactors and are flexible in use. The major drawbacks of the systems described above are the limited number of samples taken per module and the lack of automation (the disconnection of sampling containers must currently be done manually). It might be advantageous to develop plastic versions of the trace system as disposable filtration units.

3 Direct Optical Sensing

The measuring principles for optical sensors are based on the interaction between electromagnetic waves and molecules. Optical sensing is noninvasive, continuous, and not disturbed by electromagnetic fields. Parallel measurement of different process parameters is often feasible. And, since optical sensors do not have any time delay, real-time monitoring is enabled. Finally, spectroscopic measurements allow several analytes to be detected simultaneously.

Optical sensors can be coupled to disposable reactors through a transparent observation window [17]. The optical detector can be interfaced to the reactor via
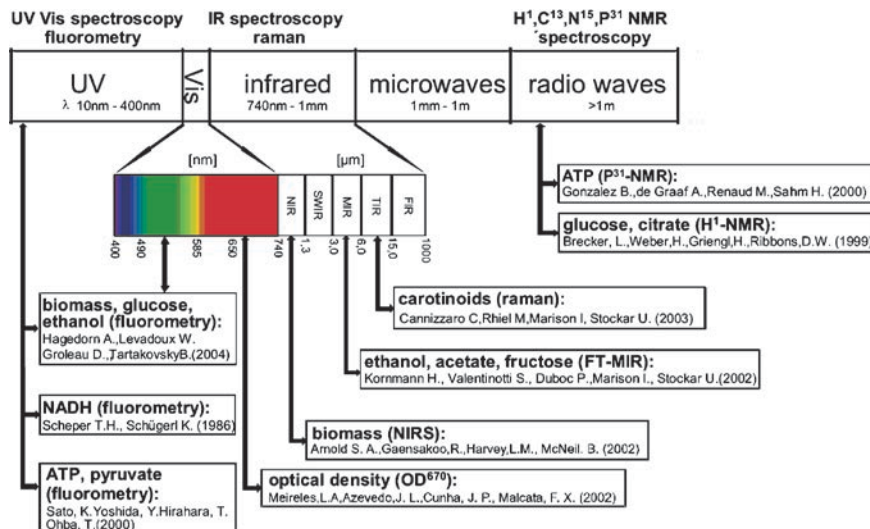
Fig. 1 Electromagnetic spectrum and application in optical measurements for biotechnological process parameters

glass fibers and thereby be physically separated, allowing the expansive analytic system to be reused. Thus, optical sensors can be applied in situ or in an online configuration. The latter might be required to avoid interference from bubbles or to provide a specific optical configuration (e.g., absorbance across a certain distance).

Figure 1 provides an overview of different spectroscopic methods and the analytes that can be detected. This review focuses on fluorimetry and IR-spectroscopy.

3.1 Fluorometry

Fluorescence sensors are applied in research as well as in industrial applications [18, 19]. Interfacing to disposable reactors can be realized via a transparent observation window to provide either in situ or online sensing. Some fluorescence sensors are optimized for measurements of NAD(P)H and use one pair of emission and excitation wavelengths [20–46]. Such NAD(P)H sensors have been used for both biomass estimation and for tracking physiological changes such as the transition between aerobic and anaerobic cell metabolisms [47].

The use of 2D process fluorometers enables the simultaneous measurement of several analytes by scanning through a range of excitation and emission wavelengths. Examples include proteins, vitamins, coenzymes, biomass, glucose, and metabolites such as ethanol, ATP, and pyruvate [48–77]. Thus, improved controlling and
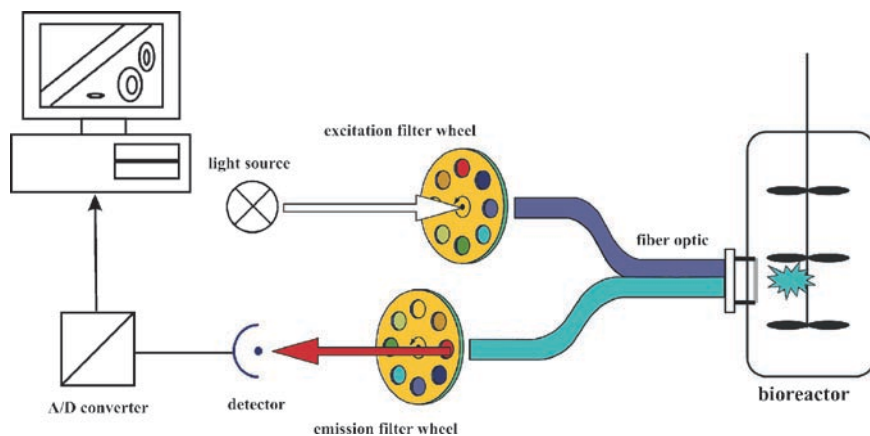
Fig. 2 General setup of the BioView 2D process fluorometer (both filterwheels are displayed with 8 filters; in reality both wheels contain 16 filters)

modeling of fermentation processes is possible. Such systems can be attached directly to the fermenter via a fiber-optic light guide so that both in situ and online measurements are possible. With 2D process fluorometers like the BioView system (DELTA Light and Optics, Hørsholm, Denmark) shown in Fig. 2, all fluorophors contained in a sample or a cell culture broth can be detected simultaneously. For that purpose 2D spectra in the range of 280-700 nm are recorded in 1-min cycles by scanning the extinction and emission wavelengths with color filters. The difference in wavelength between the filters is 10 nm.

Fluorescence spectra of fermentation broths are often complex and contain overlapping peaks. Thus, an accurate calculation of process parameters is difficult, and multivariate analysis processes like principal component analysis and neural networks must be applied [78–85].

3.2 Infrared Spectroscopy

Since concentrations of substrates like ethanol, glucose, and fructose can be calculated from IR spectra, infrared spectroscopy is of great interest for bioprocess monitoring. Bioprocesses generally take place in the aqueous phase. Due to the high IR absorbance of water (>2,500 nm), transmission spectroscopy can only be performed with short optical path length or in the near- to short-wave IR range (NIR-SWIR, 700–2,500 nm) [86]. Instead, ATR-IR-spectroscopy is more commonly used [87, 88]. The measuring principle is based on the phenomenon that during total reflection of light at an interface of two phases with different refraction indexes the light beam penetrates into the medium with the lower refraction index in the dimension of one wavelength (Fig. 3).
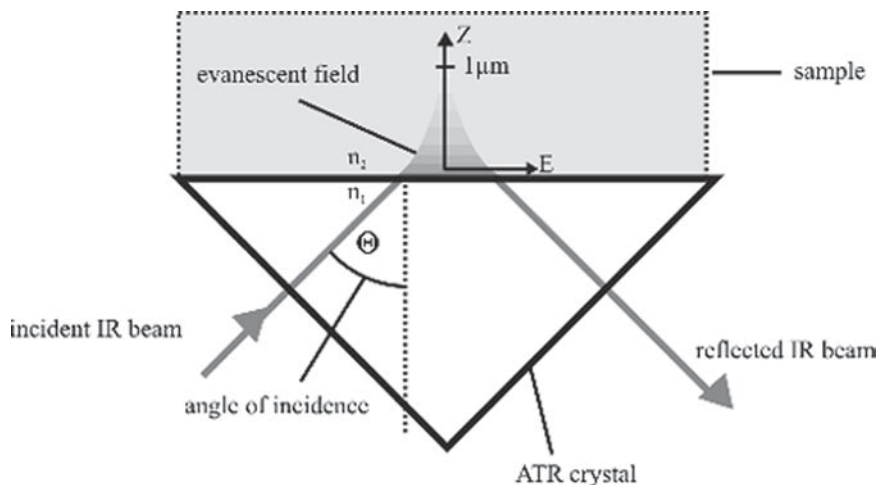
Fig. 3 Schematic diagram of the attenuated total reflection

This phenomenon is called an "evanescent wave," because the amplitude of the penetrating wave decreases exponentially with the distance to the interface. Prisms or trapezoidal-shaped crystals of germanium, zinc selenide, or diamond are often used as the material with higher refraction index. If the sample contains an analyte that absorbs IR light, the intensity of the reflected beam will decrease. By scanning across a range of wavelengths, an IR reflection spectrum is obtained that is similar to a transmission spectrum. In addition to the well established NIR transmission probes, ATR-IR probes for bioreactors are now commercial available. These can be interfaced to common IR and NIR spectrometers via silver halide fibers. The interface to a disposable reactor could be made using an SMA connector that is integrated in the reactor wall and to which the crystal, located inside the reactor, is connected. Since the ATR crystals are expensive and would have to be replaced after each use, the high costs of such a system would be a limiting factor for this application.

Direct IR monitoring of the gas phase has long been used in bioprocessing, particularly for carbon dioxide concentrations as a means to assess respiration rates. Recent developments in this area include the Capnostat 5 device, an optical CO_2 sensor for gas-phase measurements manufactured by Respironics, Inc. (Pittsburgh, USA) and commercialized via Sartorius-Stedim (Göttingen, Germany). The sensor consists of a measuring cell and a flow-through chamber (Fig. 4). The measuring principle is based on IR absorption. Infrared light is beamed through the flow-through chamber, which is connected to an exhaust port of the reactor. A photodetector measures the remaining intensity, ensuring that the measurement is independent of the radiation intensity of the light source. A batch fermentation of yeast (*Saccharomyces cerevisiae*) was monitored simultaneously with the Capnostat 5 system and with an established gas analyzer (EGAS-2, Hartmann & Braun, Frankfurt, Germany), both of which were interfaced online to the exhaust gas outlet of the bioreactor. The EGAS-2 system sampled at a frequency of 60 s and the sampling frequency of the Capnostat 5 system
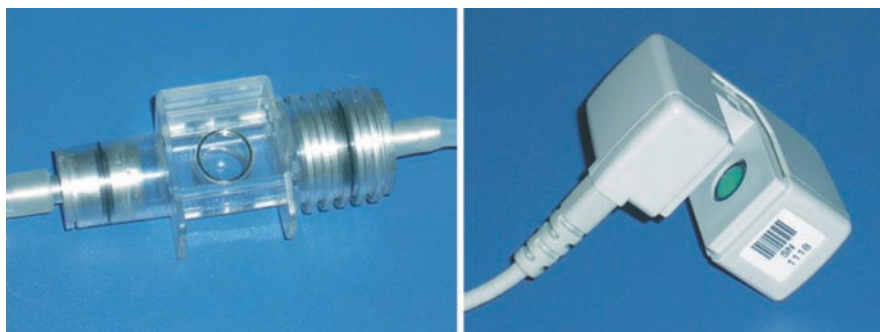
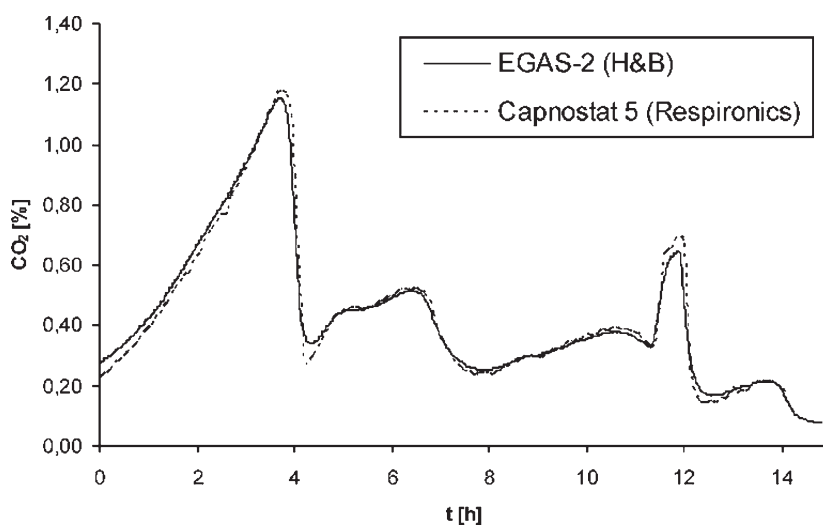**Fig. 4** Measuring cell (*left*) and sensor (*right*) of Capnostat 5

Fig. 5 CO_2-concentration in exhaust gas during a batch yeast cultivation measured with Capnostat 5 and EGAS-2

was 300 s. The reactor was aerated at 46 L h^{-1}. The CO_2 concentration was monitored for 14 h after inoculation. As shown in Fig. 5, a good correlation between the two CO_2 monitoring systems was obtained.

4 Optical Chemosensors

As described above, optical sensors detect the spectroscopic properties of the analyte itself. However, such direct measurements are often not possible. Instead, optical chemosensors, which rely on indicators with optical properties (e.g., photoluminescence, absorption, reflection) that depend on the analyte, can be used.

Optical chemosensors ("optodes") are very well suited for disposable bioreactors. The optical detector and the transducer can be interfaced via glass fibers, with the transducer placed inside the reactor as an expendable item and the external measuring equipment reused. The coupling can be realized through a transparent observation window [89]. In this manner, optical chemosensors can be implemented in either an in situ or an online format.

4.1 Optical Oxygen Sensors

The general setup of a fiber-optic oxygen sensor is shown in Fig. 6, and the whole sensing system is depicted in Fig. 7.

The measuring principle of optical oxygen sensors is based on fluorescence quenching by molecular oxygen [90–94]. A fluorescent dye is immobilized and attached to the end of the optic fiber, and an excitation light source (e.g., LED) is interfaced to the other end of the fiber. The lifetime and intensity of fluorescence depend on the oxygen concentration in the environment around the dye. The emitted fluorescence light is launched into the optic fiber, separated from the reflected excitation light by a dichroic mirror, and measured with a photomultiplier or a photodiode. Metal complexes immobilized in polymers, such as Tris-4,7-diphenyl-1,10-phenanthrolin-ruthenium(II) in silicone [20], are commonly used for optical sensing of oxygen. To avoid cross-sensitivity to ions that can also quench the fluorescence of these metal complexes, hydrophobic matrices are used for immobilization [95].

In comparison to the Clark electrode, oxygen sensors offer important advantages. First of all, optical sensors can be miniaturized. Thus, measurements with high spatial
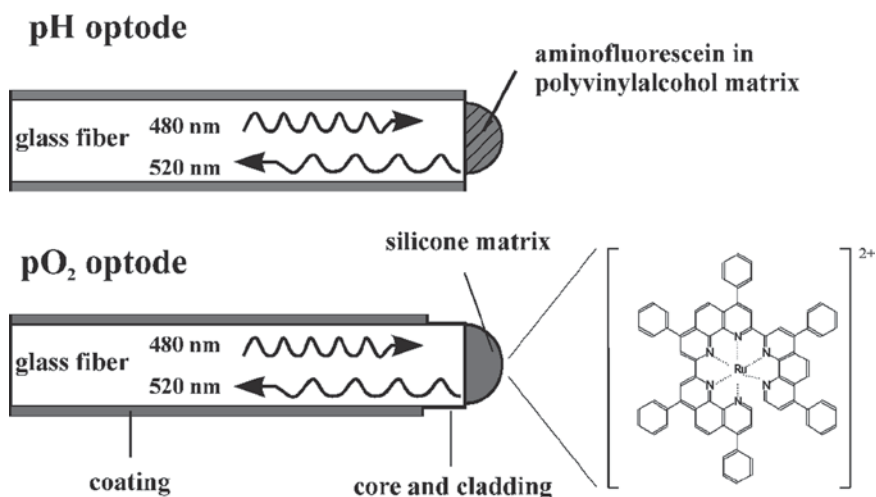
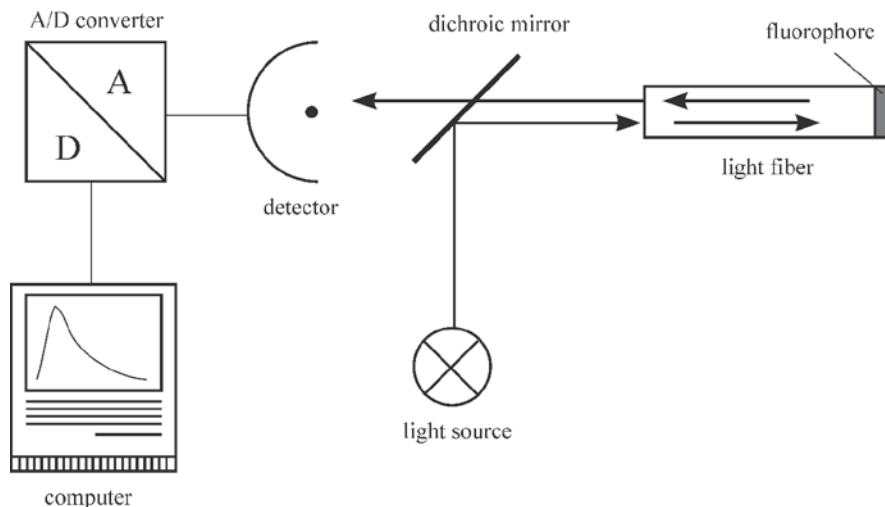**Fig. 6** pH and pO$_2$ optodes

Fig. 7 General setup of a fiber-optic sensor

resolution and in small volumes are possible [96]. Also, optical sensing is a nonreactive method, and thus measurements can be performed in diffusion limited zones, where the use of a Clark electrode would decrease the oxygen concentration. Finally, measurements are possible in both gas and liquid phases. The commercially available optical oxygen sensors (e.g., Fibox (PreSens, Regensburg, Germany), Foxy (Ocean Optics Inc., Dunedin, Florida, USA)) can be autoclaved without loss of sensitivity.

A disadvantage of optical sensors is the fact that their long-term stability is limited by photobleaching. Lifetime measurements are less affected by this phenomenon than are intensity measurements, but are technically more complex to perform [97]. The photostability of the indicator can be enhanced by specific chemical modifications. For example, multiple fluorinated platinum porphyrin is photobleached 10–20 times more slowly than the nonsubstituted dye [98]. Another approach to avoid a drift of the signal is ratiometric measuring [99].

4.2 Optical pH Sensors

For fiber-optic pH measurements, both fluorescence- and absorption-based pH indicators can be applied. Frequently used fluorescing dyes include fluoresceine derivatives and 8-hydroxy-1,3,6-pyrene trisulfonic acid [100–102], and phenol red and cresol red are common examples of indicators for absorption-based measurements [103]. The general setup of a fiber-optic pH-sensor is illustrated in Fig. 7. Like other chemosensors, these systems have the advantage of miniaturizability, and a pH sensor with a diameter less than 1 μm has been described [104]. Optodes of that scale have response times in the range of milliseconds and enable intracel-

lular measurements [105]. Disadvantages of fluorescence based pH sensors are a limited measuring range (about three pH units) and cross sensitivity to ionic strength [106]. In addition, dyes that are embedded in the polymer matrix lose their sensitivity during autoclaving or are washed out. Recent results indicate that covalently bound dyes are more stable against temperature and elution [107]. The influence of ionic strength on the signal could be minimized by immobilization of dyes in matrices that have a high ionic strength themselves.

4.3   Optical pCO_2-Sensors

Most fiber-optic pCO_2 sensors follow the same measuring principle as the Severinghaus-electrode [108, 109]. This sensor consists of a pH and a reference electrode in contact with a carbonate buffer solution on the electrode surface, embedded in a CO_2-permeable membrane. The pH value of the carbonate buffer, which is in equilibrium with the CO_2 partial pressure across the membrane, is measured. When the CO_2 concentration increases, carbon dioxide diffuses through the membrane and changes the pH value via the proton–carbonate equilibrium as described by the Henderson–Hasselbach equation. The pH change can be measured electrochemically, as is the case with the Severinghaus-electrode, as well as with optical pH sensors. Since equilibration between buffer and medium across the membrane takes place slowly, the reaction time of the sensors is in the region of minutes. The low temperature stability of noncovalently bound dyes that is observed for fluorescence based pH sensors is also valid for pCO_2 chemosensors. Due to the ionic strength dependence of the optical measurements, the carbonate buffer must be replaced frequently. A new approach makes use of a quaternary ammonium hydroxide in place of the bicarbonate buffer solution. The sensor membrane contains ion pairs consisting of an anionic pH indicator dye anion and a quaternary ammonium cation, and an additional amount of quaternary ammonium hydroxide is included. Such sensors have shorter response times and are less sensitive to ionic strength [110, 111].

5   In Situ Microscopy

The concept of in situ microscopy was first developed by Suhr et al. [112] and is based on a fully autoclavable light microscope that can be placed into a bioreactor through a 25-mm sideport. With this device, images can be acquired from the cultivation broth inside a bioreactor and an automated analysis of cell concentration and morphology may be performed. The setup of the in situ microscope and the measuring principle is illustrated in Fig. 8. It is a transmitted-light bright-field microscope with a finite-corrected objective (4×, 10×, 20×). The light source is a bright LED (12 cd, 510 nm) and images are recorded by a monochrome CCD camera. The microscope consists of separable optical and reactor segments.
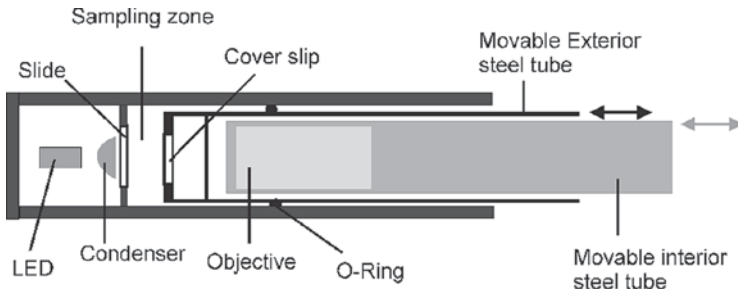
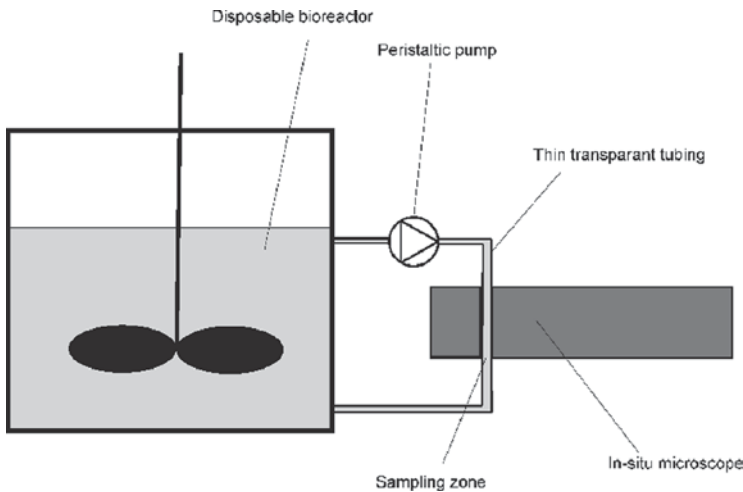**Fig. 8** Schematic setup of the in situ microscope

Fig. 9 Use of in situ microscope in a bypass through a thin transparent tubing

The reactor segment can be placed into a bioreactor and contains the sampling zone which is bordered by two sapphire windows. The cells passing through the sampling zone are visualized by the CCD-camera. This enables an automated in situ measurement of cell parameters. The optical segment consists of a linear table with two moveable slides, which are controlled by two stepper motors. One of the slides is connected to a tube that regulates the height of the sampling zone, controlling the flow through the sampling zone. The second slide is connected to the objective and is used for focusing the image. The in situ microscope has been successfully applied in the monitoring of yeast [113, 114], mammalian [115–117], and microcarrier cultivations [118].

The use of an in situ microscope as a disposable system has not yet been examined; however, two approaches can be envisioned. The first requires a bypass containing a section of thin, transparent tubing. The tubing would be placed inside the sampling zone of the microscope and sample would be pumped through this zone (Fig. 9). The basic advantage of this approach is that the existing in situ
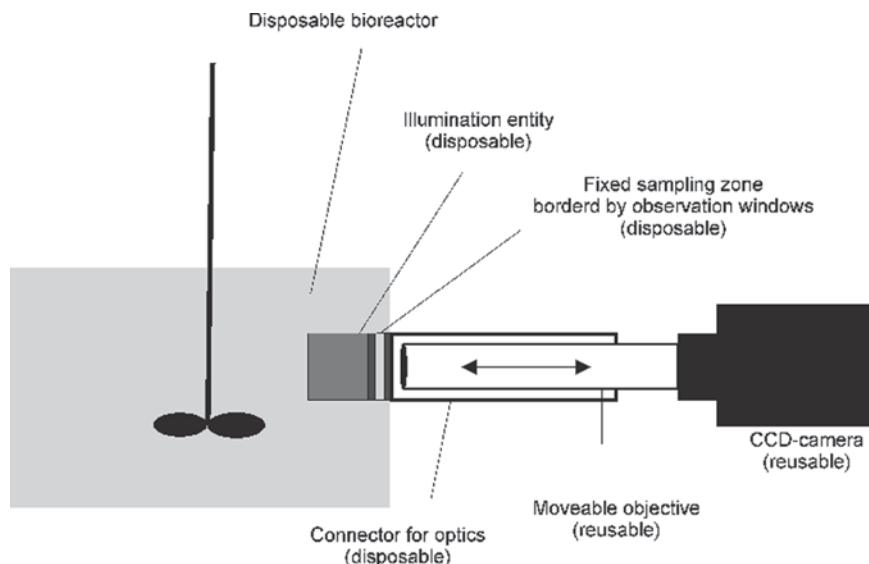
Fig. 10 Possible setup of a disposable in situ microscope

microscope could be applied without any changes to the hardware. Challenges may arise from an unstable flow through the tubing and difficulties focusing the cells in the tubing.

The second approach requires a complete redesign of the entire reactor segment of the in situ microscope (Fig. 10). The illumination section containing the LED, the condenser lens, and two glass windows could be constructed to be disposable and may be integrated and sterilized with the disposable reactor. This would lead to a system with a fixed sampling zone, and thus modules with different heights are required depending on the cell type. The objective and the CCD camera, which are the expensive parts of the microscope, are reusable and may be connected to the disposable module from outside for focusing.

6   Electrochemical Sensors

Thick- and thin-film sensors as well as ion-sensitive field-effect transistors belong to the class of electrochemical sensors with potential as disposable sensors in bioprocess control. This fact derives from the fact that these sensors are small and can be produced inexpensively and in large quantities. This chapter only covers the area of ion-sensitive field-effect transistors since they are very well suited for disposable bioreactors.

6.1 Principles of Ion-Sensitive Field-Effect Transistors (ISFETs)

ISFETs are chemical sensors that rely on silicon as a base material. Historically, their first applications were in biomedical measurements, due to their small dimensions. Wise at al. designed silicon needles carrying microelectrodes and an integrated amplifier circuitry for electrophysiological measurements [119]. Bergveld et al. [120] proposed to replace the amplifying junction field-effect transistor with a metal oxide semiconductor FET (MOSFET) without gate metallization. To shorten the connection between the microelectrode and the amplifier, it was necessary to have the bare gate of the MOSFET in direct contact with the biological environment, containing a reference electrode that can be viewed as a remote gate metallization (Fig. 11). The resulting device responded to ion activities, giving birth to the ISFET. The ISFET connects the attributes of semiconductors with the benefits of chemically sensitive glass-membrane electrodes [121, 122]. The chemical sensitivity derives from the attachment of ions to the gate membrane leading to an electric potential that changes the conductivity and thus current between source and drain electrode.

These changes can be measured electrically and are proportional to the activities of the detected ions. There are two different possible modes of operation for an ISFET:

1. Gate and drain voltage U_{Gate} and U_{Drain} are kept constant; measurement of drain current I_{Drain}
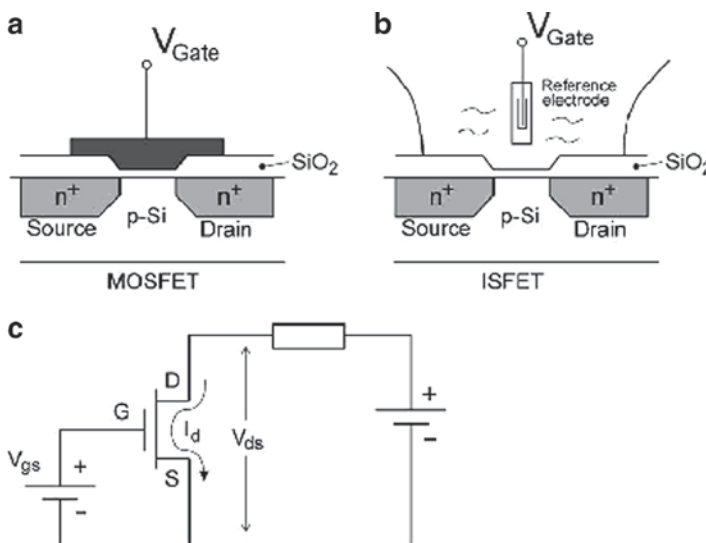2. I_{Drain} and U_{Drain} are kept constant; measurement of actively compensated U_{Gate}

Fig. 11 Schematic of MOSFET (**a**), ISFET (**b**), and electric diagram (**c**)

The second mode has the advantage that a change in the gate's boundary potential is measured directly. Due to the superior electrical properties of the Si–SiO$_2$-interface, SiO$_2$ is a standard gate insulator in the manufacturing of MOSFETs. However, SiO$_2$ exhibits a low sensitivity for the detection of H$_3$O$^+$ concentration (i.e., pH). Thus, Si$_3$N$_4$, Al$_2$O$_3$, Ta$_2$O$_5$, and ZrO$_2$ have been introduced as alternative gate materials. These materials show increasing pH sensitivity in the order SiO$_2$ <Si$_3$N$_4$ <Al$_2$O$_3$ <Ta$_2$O$_5$. Usually, pH-sensitive ISFETs – also referred to as pHFETs – are fabricated with two dielectric materials in the gate region: SiO$_2$ on silicon and a second dielectric material on the SiO$_2$ with direct contact to the electrolyte. The pH-response of pHFETs can be determined by the site-binding model [123] and its further extensions [124–126] describing the charging mechanisms of the interface between the gate-insulator and electrolyte, which depend on the H$^+$ activity in the bulk solution. For a Ta$_2$O$_5$ pHFET at ambient temperature a theoretical slope of 58 mV per pH unit can be calculated.

ISFETs can be sensitized for a wide variety of analyte molecules by modification of their gate region. Attachment of thin membrane layers on the ISFET gate that contain ionophores (e.g., crown ether) sensitize the FET for potassium ions [127]. Immobilization of enzymes on the gate of a pHFET that deliver or consume protons in their catalyzed reaction can be used for the construction of EnFET biosensors. As there is a wide variety of enzymes with reaction mechanisms that are directly compatible with pHFET transducers (or with mechanisms that could be properly modified) many EnFETs have been described in the literature [128–143]. Enzymes are not the only means to achieve biological recognition of an analyte, and thus the immobilization of antibodies (ImmunoFET), living cells, and nucleic acids (DNAFET) have been reported [144–146].

6.2   pHFETs in Bioreactor Monitoring

ISFETs and especially pHFETs have good potential as disposable sensors in bioreactor monitoring, but several challenges must be overcome before they can be applied. Their main advantage is the low price and high quantity in which they can be produced, which derives from well established silicon microelectronics technologies. Furthermore the application of standard CMOS processes enables simple combination of ISFETs with CMOS electronics [147, 148]. A second advantage is their ability to be miniaturized [149]. ISFETs can be small enough to measure in very small volumes, and thus microreactors or even 96-well plates for screening purposes can be easily equipped with pHFETs for monitoring or process control [150, 151]. Another desirable attribute of pHFETs is the impedance transformation on the point of measurement, which allows electrical connections between sensor and transmitter to be very long. Since pHFETs housings can be fabricated from polymers, they can be applied in pharmaceutical or food and beverage processes without concern for glass fragments. In addition, pHFETs can be mounted horizontally or even overhead, whereas conventional multiuse pH electrodes require minimum

mounting angles of 15°. Finally, pHFETs can be applied to processes at subzero temperature levels, in contrast to standard glass electrodes that rely on hydrolysis of glass materials and thus possess significantly slow kinetics and long response times at low temperatures.

Several major problems have prevented a pHFET breakthrough to the market [152]:

1. Light sensitivity of early ISFET sensors, long- and short-term drift and sub Nernstian response resulting in decreasing performance
2. Lack of proper all solid-state reference electrodes
3. Poor packaging integrity due to the difficulties with reliable encapsulation of sensor chips
4. Sensitivity of gate materials against cleaning in place (CIP) processes [153]

The first problem has been overcome by the introduction of improved gate materials like Ta_2O_5, ZrO_2, Al_2O_3, or even combinations of these materials [154, 155]. This has led to pHFETs with signal drift characteristics that are comparable to glass pH electrodes.

Considerable effort has been devoted to the development of miniaturized solid-state reference electrodes with longer stability but so far none of the designs match the properties of conventional Ag/AgCl-electrodes [156]. Most desirable for mass production of disposable pHFETs would be a reference electrode compatible with wafer production of the sensors. Thus far, attempts to create these electrodes by means of thin film technologies, without any inner electrolyte, have suffered from unstable potentials and unwanted cross-sensitivity towards anions and redox-reactions, resulting from the direct contact of the "quasireference" electrode to the analyte solution. Typical lifetimes for thin-film Ag/AgCl quasireference electrodes from several minutes to 10 h have been reported [157, 158]. Coverage of thin-film Ag/AgCl electrodes with KCl-saturated gels and the use of additional membranes against KCl leaching has further improved achievable lifetimes but is still unsatisfactory, as corrosion of the Ag/AgCl thin film still strongly limits its durability. The best results have been achieved by miniaturization of the standard reference electrode, primarily by means of screen-printed Ag/AgCl thick films of a few hundred microns covered by KCl-saturated gel-like membranes and additional protective membranes. A different approach is the use of a noble metal pseudoelectrode in addition to a second ISFET, which has been made insensitive to protons, as a reference electrode (REFET) [159–161]. The pHFET/REFET combination can easily be miniaturized and processed with standard on-wafer CMOS techniques, but requires additional steps for passivation of the REFET gate [162] and the preparation of the noble metal pseudoelectrode.

The encapsulation and the bonding of ISFET sensors pose further challenges. As only the gate area (including the reference electrode) must have contact to the liquid phase, the rest of the chip and even the vicinity to the gate must be properly sealed from the corrosive liquid. Encapsulation techniques for commercialized multiuse pHFET sensors from Endress +Hauser, Sentron, Honeywell and Mettler Toledo have been reviewed [163]. Since these multiuse sensors are intended to

replace standard glass pH electrodes, they all have form factors very similar to electrodes with macroscopic Ag/AgCl-reference electrodes. The materials for encapsulation should be highly resistant to leaking, chemical and electrical strain, and possess either similar thermal coefficients of expansion or an encapsulation design that compensates a mismatch. The encapsulation scheme must also be compatible with the integration of a reference electrode.

Since production lines in the pharma, biotechnology, and food industries must be regularly cleaned and sterilized by means of standardized CIP and sterilization in place (SIP) procedures, sensors should endure these procedures preferably without any decrease in their performance. As standard CIP procedures include the application of NaOH solutions at elevated temperatures, this has restricted the application of ISFETs. A gate material like Si_3N_4 is unstable under these conditions and dissolves in hot alkaline solutions. Recently, Ta_2O_5 has been used with success as the gate material as it is quite stable during SIP processes and also during CIP with hot caustic solutions [164].

For disposable sensors, some of the requirements for multiuse ISFET sensors and their reference electrodes may be reduced. Disposable sensors integrated into disposable reactors must only fulfil the requirements associated with a single operation for a limited amount of time (hours to days). Until now, only disposable pHFET sensors for medical applications have been commercialized. Catheters with an integrated pHFET and a standard reference electrode for 24 h-monitoring of esophagus, stomach, and duodenum are available. The manufacturer specifies a resolution of 0.1 pH units and a typical drift of 0.1 pH units in 24 h [165].

7 Conductivity Sensors

Electrolytic conductivity is an important parameter in many aspects of bioprocessing. Conductivity as a measure of the concentration of ions in solution and thus purity is a critical quality parameter. As such, it is applied to the preparation of fermentation media, the formulation of biotechnological products like vaccines or therapeutic proteins with ultrapure water, the chromatographic purification of biotechnological products, tangential flow filtration, ultrafiltration, reverse osmosis, and CIP. Conduc-tivity is also an interesting parameter to monitor in disposable bioreactors.

The principle of operation of a conductivity sensor is well known. The simplest embodiment comprises an a.c. voltage source applying a sine- or square-wave voltage of some kHz to an electrode with a parallel second electrode some distance away. The space between is filled with the analyte solution. By measuring the current through and the voltage drop across the electrodes, the resistance can be calculated according to Ohm's law. Because the voltage drop depends on geometry, surface parameters, and material of the electrodes, the system has to be calibrated with a solution of known conductivity. The relation between the observed resistance R and conductivity κ is given by the cell constant $\kappa_{cell} = \kappa R$. Electrode polarization that

arises from the formation of an electrical double layer between electrode and the ions in solution can cause significant measurement inaccuracies. A solution to this problem was the introduction of the four-electrode system. The outer two of four parallel electrodes are connected to the a.c. source and carry the current, while the inner two electrodes measure the voltage drop and are driven currentless and thus free of polarization. Usually the voltage drop over the inner electrodes is held constant by regulation of the voltage source. The current through the system is the signal that correlates with conductivity. As ion mobility and thus conductivity increases with increasing temperature, conductivity must be compensated for temperature changes. By consensus, conductivity is related to 25 °C and compensated to this temperature. Therefore, conductivity probes are usually also outfitted with a temperature sensor. Although there are many multiuse conductivity probes on the market, until now only Scilog [166] has come up with a disposable system. The disposable flow-through cell, with an integrated temperature sensor, is precalibrated and stores the sensor constants within an EPROM. The system is certified to be GMP compliant.

8 Sensors Based on Ultrasound

Techniques based on ultrasonic measurements have been applied in fields such as the process industry and medicine. Ultrasonic signals are used for level detection and flow measurements to monitor and control production processes. In bioprocess engineering, ultrasound is used to measure the concentration of substances that alter sound velocity and acoustic impedance [167], and can also be used to mix, emulsify, or suspend particulates in a reaction broth [168]. Becker et al. used the ultrasonic signals to determine the extract content in fermenting beer [169]. They applied artificial neural networks to compensate the temperature influence. The impacts of sonic treatment on both aerobic and anaerobic digestion are presented by Khanal et al. [170]. They point out that ultrasonic density is more important than sonication time for efficient sludge disintegration. Furthermore, ultrasonic signals can be employed to enhance the activity of immobilized enzymes [171] or to nebulize nutrient solution into microdroplets 1 μm in diameter [172]. Ultrasonic signals are usually produced using a piezo crystal with a frequency range from 20 kHz to 1 GHz: however, the upper bounds can be as much as three orders of magnitude higher for special applications. New hardware developments based on the tremendous progress in electronics are described by Henning and Rautenberg [167]. These systems take advantage of digital data processing methods.

Ultrasonic sensors normally have excellent long-term stability, a fast response, and low power consumption [173]. No reagents are necessary to use these sensors for monitoring. The main advantage of ultrasonic analysis and processing is that it can be interfaced to the process noninvasively in either an in situ or an online device. The transmitter and receiver are placed at the outside of a reactor as can be seen in Fig. 12. Due to the fact that both the velocity and the attenuation of the signal will depend specifically on substances in the reactor, both variables are measured.
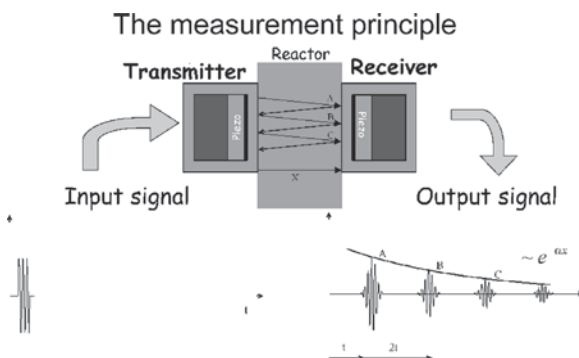
Fig. 12 The scheme of an ultrasonic sensor for bioreactor monitoring

An ultrasonic burst is sent by the transmitter into the reactor from the outside. The signal passes through the liquid and is measured with its echo by the receiver (Fig. 12). The attenuation of the signal can be determined by the exponential decrease of the signal amplitude. Using the time differences of the pulses, the velocity can be calculated. The velocity of the ultrasonic wave c_{US}, is related to the density of the liquid according to

$$c_{US} = \sqrt{\frac{1}{\kappa\rho}} \tag{1}$$

with κ being the adiabatic compressibility and ρ the density. Because the adiabatic compressibility depends strongly on the temperature [174], temperature effects must be considered. Information about the adiabatic compressibility is hard to obtain, and therefore suitable calibration procedures are required. Furthermore, the density of the liquid under consideration will depend on substances that are often encountered in bioprocess engineering. These include dependences on cell density and glucose concentration, which are important variables for almost all cultivation processes. Knowing all the influencing variables except one, the unknown can be predicted by using this technique.

An error analysis due to thin layers of deposits on the sensor surface is given by Püttmer et al. [175]. Utilizing simulation techniques, the authors analyze the measurement errors of an ultrasonic liquid density sensor. Further limitations of the evaluation of ultrasonic signals are the dependence of acoustic properties on the specific substance concentration, the nonmonotonic velocity (depending on concentration and temperature), limited resolution of adsorption and impedance measurement, strong influence on temperature, and the sensitivity to air bubbles [167].

A disposable bioreactor can be equipped with an ultrasonic measurement device without becoming very expensive. Schneditz et al. applied a sound–speed sensor to determine total protein concentration in a disposable blood-perfused tube [176]. They used as the disposable system a flexible tube, which was pushed into the gap of the acoustic support where the transducer is mounted to the walls opposing the gap. The dependence of the sound velocity on temperature and total protein concentration is considered by a polynomial.

Brown and Mason [177] presented an evaluation of polyvinylidene fluoride as a material of interest for ultrasonic transducers. They showed that the same disposable transducers can be used for both ultrasonic and acoustic emission to enable nondestructive testing. For the disposable polyvinylidene fluoride film transducers, quantitative ultrasonic- and vibration-sensing performance was demonstrated, illustrating the potential of this technique for monitoring a disposable bioreactor.

9 Conclusion

The variety of sensor systems for bioprocess monitoring is huge. Several commercial systems are available. Although the number of disposable sensors is still low, the concepts used for standard bioreactor systems can often easily be transferred to disposable systems (Table 1). In particular, optical sensors and semiconductor devices offer advantages. Significant advances must still be made so that disposable sensors achieve the same validity as those used in conventional bioprocess monitoring. The data handling and chemometrics must also be improved and it is likely that knowledge-based systems will be used extensively in the future.

Table 1 Process variables used for bioprocess analysis and possible disposable in situ sensing concepts. The status of each concept is indicated as commercially available (CA), in research (IR), or not available (NA)

Variable	In situ sensing concept	Disposable system (problems)
Temperature	Semiconductors	CA
	Optical	IR
Conductivity	Semiconductors	IR
pH	Semiconductors (ISFETs)	NA (reference electrode needed)
	Optical	IR
pO_2	Optical	CA
pCO_2	Optical (fluorescence)	IR
	Optical (IR)	IR
Glucose, ethanol, lactate	Optical (ATR-IR)	NA (ATR crystal too expensive)
	Ultrasonic	NA bypass needed
Cell density/biomass	Optical (in situ microscope)	NA microscope too expensive
	Ultrasonic	NA bypass needed

References

1. Novais J, Titchener-Hooker N, Hoare M (2001) Biotechnol Bioeng 75:143
2. Farid S, Washbrook J, Titchener-Hooker N (2005) Biotechnol Bioeng 21:486
3. Chirvase AA, Dumitrache I, Caramihai MD (2007) Rev Roum Chim 52:621
4. Becker T, Hitzmann B, Muffler K, Portner R, Reardon KF, Stahl F, Ulber R (2007) Adv Biochem Eng Biotechnol 105:249
5. Junker BH, Wang HY (2006) Biotechnol Bioeng 95:226
6. Rhee IL, Ritzka A, Scheper T (2004) Biotechnol Bioprocess Eng 9:156
7. O'Flaherty B (2004) Gen Eng News 24:54
8. Schugerl K (2001) J Biotechnol 85:149
9. Scheper T, Hitzmann B, Stark E, Ulber R, Faurie R, Sosnitza P, Reardon KF (1999) Anal Chim Acta 400:121
10. Rehbock C, Beutel S, Brückerhoff T, Hitzmann B, Riechers D, Rudolph G, Stahl F, Scheper T, Friehs K (2008) Chem Eng Technol 80:267
11. Ulber R, Hitzmann B, Scheper T (2001) Chem Eng Technol 73:19
12. Hilmer JM, Scheper T (1996) Acta Biotechnol 16:185
13. http://www.grotonbiosystems.com/pressroom/press_docs/MKT-046_ProductBulletin_SampleProbe.pdf. Accessed 27 May 2008
14. Kinney S, Phillips C (2007) BioProcess Int 5:52
15. http://www.cellexusbiosystems.com/index_files/Cellexussampler.pdf. Accessed 20 Mar 2008
16. Furey J, Proulx S (2007) US Patent 7,293,477 B2
17. Scheper T et al. (1999) Anal Chim Acta 400:121
18. Marose S et al. (1999) Trends Biotechnol 17:30
19. Schugerl K (2001) J Biotechnol 85:149
20. Wolfbeis OS et al. (2005) J Mater Chem 15:2657
21. Scheper T, Schugerl K (1986) J Biotechnol 3:221
22. Ulber R et al. (2003) Anal Bioanal Chem 376:342
23. Scheper T, Gebauer A, Kuhlmann W, Meyer HD, Schügerl K (1984) DECHEMA-Monographien, Verlag Chemie, 95:83
24. Scheper T, Gebauer A, Sauerbrei A, Niehoff A, Schügerl K (1984) Anal Chim Acta 163:111
25. Scheper T, Schügerl K (1986) J Biotechnol 3:221
26. Scheper T, Schügerl K (1986) Chem Eng Technol 58:433
27. Scheper T, Schügerl K (1986) Appl Microbiol Biotechnol 23:440
28. Schügerl K, Lorenz T, Lübbert A, Niehoff J, Scheper T, Schmidt W (1986) Trends Biotechnol 4:11
29. Reardon KF, Scheper T, Bailey JE (1986) Biotechnol Lett 8:817
30. Gebauer A, Scheper T, Schügerl K (1987) Bioprocess Eng 2:13
31. Scheper T, Gebauer A, Schügerl K (1987) Chem Eng J 34:B7
32. Scheper T, Lorenz T, Schmidt W, Schügerl K (1987) Ann N Y Acad Sci 506:431
33. Reardon KF, Scheper T, Bailey JE (1987) Chem Eng Technol 59:600
34. Schügerl K, Lübbert A, Scheper T (1987) Chem Eng Technol 59:701
35. Reardon KF, Scheper T, Bailey JE (1987) Biotechnol Progress 3:153
36. Müller W, Wehnert G, Scheper T (1988) Anal Chim Acta 213:47
37. Müller W, Anders KD, Scheper T (1989) Chem Eng Technol 61:564
38. Scheper T, Bückmann FA (1990) Biosens Bioelectron 5:125
39. Wehnert G, Anders KD, Bittner C, Kammeyer R, Hübner U, Niellsen J, Scheper T (1990) Chem Eng Technol 6:211
40. Scheper T (1990) Biotech Forum Eur 7:67
41. Reardon KF, Scheper T, Anders KD, Müller W, Bückmann AF (1990) Appl Biochem Biotechnol 24/25:363
42. Schügerl K, Lübbert A, Scheper T (1990) Int Chem Eng 30:433

43. Scheper T, Brandes W, Grau C, Hundeck HG, Reinhardt B, Rüther F, Plötz F, Schelp C, Schügerl K, Schneider KH, Fiffhorn F, Rehr B, Sahm H (1991) Anal Chim Acta 249:25
44. Plötz F, Schelp C, Anders KD, Eberhardt F, Scheper T (1991) Proc SPIE 1510:224
45. Scheper T (1992) J Ind Microbiol 9:163
46. Anders KD, Aknoukh R, Scheper T, Kretzmer G (1992) Chem Eng Technol 64:572
47. Rhee JI et al. (2005) Biochem Eng J 22:135
48. Hagedorn A et al. (2004) Biotechnol Progr 20:361
49. Sato K et al. (2000) J Biosci Bioeng 90:294
50. Anders KD, Wehnert G, Thordsen O, Scheper T, Rehr B, Sahm H (1993) Sens Actuators B 11:395
51. Thordsen O, Lee SJ, Degelau A, Scheper T, Loos H, Rehr B, Sahm H (1993) Biotechnol Bioeng 42:387
52. Müller C, Kohls O, Comte A, Scheper T (1993) Chem Eng Technol 65:1086
53. Schügerl K, Bellgardt KH, Kretzmer G, Hitzmann B, Scheper T (1993) Chem Eng Technol 65:1447
54. Marose S, Lindemann C, Ulber R, Scheper T (1999) Trends Biotechnol 17:30
55. Scheper T, Lammers F (1994) Curr Opin Biotechnol 5:187
56. Mukherjee J, Lindemann C, Scheper T (1999) Appl Microbiol Biotechnol 52:489
57. Tartakovsky B, Sheintuch M, Hilmer JM, Scheper T (1996) Biotechnol Progr 12:126
58. Scheper T, Hilmer JM, Lammers F, Müller C, Reinecke M (1996) J Chromat A 725:3–12
59. Knüttel T, Hartmann T, Meyer H, Scheper T (2001) Enzyme Microb Technol 29:150
60. Müller C, Hitzmann B, Schubert F, Scheper T (1997) Sens Actuators B 40:71
61. Marose S, Lindemann C, Scheper T (1998) Biotechnol Progr 14:63
62. Schügerl K, Lindemann C, Marose S, Scheper T. (1998) Bioprocess Eng Course 400
63. Pekeler T, Lindemann C, Scheper T, Hitzmann B (1998) Chem Eng Technol 70:1610
64. Marose S, Lindemann C, Ulber R, Scheper T (1999) Trends Biotechnol 17:30
65. Lindemann C, Marose S, Scheper T, Nielsen HO, Reardon KF (1999) Encyclopedia of bio-process technology: fermentation, biocatalysis and bioseparation. Wiley, New York, p 1239
66. Mukherjee J, Lindemann C, Scheper T (1999) Appl Microbiol Biotechnol 52:489
67. Scheper T, Hitzmann B, Stärk E, Ulber R, Faurie R, Sosnitza P, Reardon KF (1999) Anal Chim Acta 400:121
68. Ulber R, Hitzmann B, Scheper T, Reardon KF (2000) Enyclopedia of Microbiology, vol. 1. Academic, New York, p 567
69. Knüttel T, Hartmann T, Meyer H, Scheper T (2001) Enzyme Microb Technol 29:150
70. Ulber R, Protsch C, Solle D, Hitzmann B, Willke B, Faurie R, Scheper T (2001) Eng Life Sci 1:15
71. Stärk E, Hitzmann B, Schügerl K, Scheper T, Fuchs C, Köster D, Märkl H (2002) Adv Biochem Eng Biotechnol 74:22
72. Bartolome A, Ulber R, Scheper T, Sagi E, Belkin S (2003) Sens Actuators B 89:27
73. Sagi E, Hever N, Rosen R, Bartolome A, Premkumas JR, Ulber R, Lev O, Scheper T, Belkin S (2003) Sens Actuators B 90:2
74. Knüttel T, Meyer H, Scheper T (2005) Anal Chem 77:6184
75. Hantelmann K, Kollecker M, Hüll D, Hitzmann B, Scheper T (2005) J Biotechnol 121:410
76. Knüttel T, Meyer H, Scheper T (2006) Enzyme Microb Technol 39:607
77. Ganzlin M, Marose S, Lu X, Hitzmann B, Scheper T, Rinas U (2007) J Biotechnol 132:461
78. Tartakovsky B et al. (1996) Biotechnol Prog 12:126
79. Pekeler T et al. (1998) Chem Eng Technol 70:1610
80. Tartakovsky B, Sheintuch M, Hilmer JM, Scheper T (1997) Bioprocess Eng 16:323
81. Hitzmann B, Broxtermann O, Cha YL, Sobieh O, Stärk E, Scheper T (2000) Bioprocess Eng 23:337
82. Solle D, Geissler D, Stärk E, Scheper T, Hitzmann B (2003) Bioinformatics 19:173
83. Boehl D, Solle D, Hitzmann B, Scheper T (2003) J Biotechnol 105:179
84. Geissler D, Solle D, Stärk E, Scheper T, Märkl H, Hitzmann B (2003) Eng Life Sci 3:397

85. Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Motesinos JL, Valero F (2006) J Biotechnol 124:412
86. Arnold SA et al. (2002) Biotechnol Bioeng 80:405
87. Mazarevica G et al. (2004) Appl Spectrosc 58:804
88. Armenta S et al. (2005) Anal Chim Acta 545:99
89. Scheper T et al. (1999) Anal Chim Acta 400:121
90. Köneke R, Comte A, Jürgens H, Kohls O, Lam H, Scheper T (1998) Chem Eng Technol 70:1661
91. Kohls O, Scheper T (2000) Sens Actuators B 70:121
92. Tservistas M, Köneke R, Comte A, Scheper T (2001) Enzyme Microb Technol 28:637
93. Frerichs JG, Joeris K, Konstantinov K, Scheper T (2002) Chem Eng Technol 74:1629
94. Joeris K, Scheper T (2003) J Colloid Interf Sci 267:369
95. Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Motesinos JL, Valero F (2006) J Biotechnol 124:412
96. Klimant I et al. (1997) Sens Actuators B 38:29
97. Lam HT. (2002) Dissertation, University of Hanover
98. Lai SW et al. (2004) Inorg Chem 43:3724
99. Park EJ et al. (2005) J Mater Chem 15:2913
100. Munkholm C, Walt DR (1998) Talanta 35:109
101. Mills A, Chang Q (1993) Analyst 118:839
102. Fritzsche M, Barreiro CG, Hitzmann B, Scheper T (2007) Sens Actuators B 128:133
103. Mills A, Chang Q, McMurray (1992) Anal Chem 64:1383
104. Tan W et al. (1992) Anal Chem 64:2985
105. Lu J, Rosenzweig Z (2000) Fresenius Z Anal Chem 366:575
106. Dremel BA et al. (1992) Chem Eng Technol 64:510
107. Kermis HR, Kostov Y, Rao G (2003) Analyst 128:1181
108. Severinghaus JW, Bradley AF (1958) J Appl Phys 13:515
109. Uttamlal M, Walt DR (1995) BioTechnology 13:597
110. Ge X, Kostov Y, Rao G (2003) Biosens Bioelectron 18:857
111. Weigl BH, Wolfbeis OS (1995) Sens Actuators B 28:151
112. Suhr H, Wehnert G, Schneider K, Bittner C, Scholz T, Geissler P, Jahne B, Scheper T (1995) Biotechnol Bioeng 47:106
113. Bittner C, Wehnert G, Scheper T (1998) Biotechnol Bioeng 60:24
114. Rudolph G, Bruckerhoff T, Bluma A, Korb G, Scheper T (2007) Chem Eng Technol 79:42
115. Joeris K, Frerichs J, Konstantinov K, Scheper T (2002) Cytotechnology 38:129
116. Frerichs JG, Joeris K, Konstantinov K, Scheper T (2002) Chem Eng Technol 74:1629
117. Joeris K, Scheper T (2003) J Colloid Interf Sci 267:369
118. Anton F, Burzlaff A, Kasper C, Bruckerhoff T, Scheper T (2007) Eng Life Sci 7:91
119. Wise KD, Angell JB, Starr A (1970) IEEE Trans Biomed Eng 17:238
120. Bergveld P (1968) IEEE Trans Biomed Eng 15:102
121. Bergveld P (1970) IEEE Trans Biomed Eng 17:70
122. Bergveld P (1972) IEEE Trans Biomed Eng 19:342
123. Yates DE, Levine S, Healy TW (1974) J Chem Soc 70:1807
124. Vanhal REG, Eijkel JCT, Bergveld P (1995) Sens Actuators B 24:201
125. Bergveld P, Vanhal REG, Eijkel JCT (1995) Biosens Bioelectron 10:405
126. van Hal REG, Eijkel JCT, Bergveld P (1996) Adv Colloid Interface Sci 69:31
127. Khanna VK et al. (2007) Indian J Eng Mater Sci 14:112
128. Kullick T, Ulber R (1995) In: Freitag R. (ed) Biosensors in analytical biotechnology. Landes Bioscience, Austin
129. Migita S et al. (2007) Anal Sci 23:45
130. Scheper T, Reardon KF (1991) In: Göpel W, Hesse J, Memel JN. (eds) Sensors, vol 2. VCH, Weinheim, p 1024
131. Brand U, Reinhardt B, Rüther F, Scheper T, Schügerl K (1991) Sens Actuators B 4:315

132. Kullick T, Müller C, Plötz F, Rüther F, Scheper T, Schügerl K (1993) In: Alcock SJ, Turner APF. (eds) In-vivo chemical sensors. Cranfield, Bedford, UK, p 65
133. Menzel C, Beyer M, Kullick T, Quack R, Scheper T, Schügerl K (1993) Biol Ital 23:9
134. Scheper T, Brandes W, Maschke H, Plötz F, Müller C (1993) J Biotechnol 31:345
135. Beyer M, Menzel C, Quack R, Scheper T, Schügerl K, Treichel W, Voigt H, Ullrich M, Ferretti R (1994) Biosens Bioelectron 9:17
136. Kullick T, Ulber R, Meyer HH, Scheper T, Schügerl K (1994) Chem Eng Technol 66:704
137. Sevilla F, Kullick T, Scheper T (1994) Biosens Bioelectron 9:25
138. Kullick T, Ulber R, Meyer HH, Scheper T, Schügerl K (1994) Anal Chim Acta 293:271
139. Kullick T, Beyer M, Henning J, Lerch T, Quack R, Zeitz A, Hitzmann B, Scheper T, Schügerl K (1994) Anal Chim Acta 296:263
140. Kullick T, Bock U, Schubert J, Scheper T, Schügerl K (1995) Anal Chim Acta 300:25
141. Kullik T, Quack R, Röhrkasten C, Pekeler T, Scheper T (1995) Chem Eng Technol 18:225
142. Menzel C, Lerch T, Scheper T, Schügerl K (1995) Anal Chim Acta 317:259
143. Ulber R, Scheper T (1998) Methods Biotechnol 6:35
144. Selvanayagam ZE et al. (2002) Biosens Bioelectron 17:821
145. Castellarnau M et al. (2007) Sens Actuators B 120:615
146. Song KS et al. (2006) Phys Rev E 74:041919
147. Wei JB et al. (2006) Rare Metal Mater Eng 35:443
148. Joo S, Brown RB (2008) Chem Rev 108:638
149. Bergveld P (2003) Sens Actuators B 88:1
150. Lorenzelli L et al. (2003) Biosens Bioelectron 18:621
151. Maharbiz MM et al. (2004) Biotechnol Bioeng 85:376
152. Oelssner W et al. (2005) Sens Actuators B 105:104
153. Schoning MJ et al. (2005) Sens Actuators B 111:423
154. Matsuo T, Esashi M (1981) Sens Actuators B 1:77
155. Teravaninthorn U, Miyahara Y, Moriizumi T (1987) Jpn J Appl Phys 26:2116
156. Simonis A et al. (2005) Electrochim Acta 51:930
157. Kim HR et al. (2004) Sens Actuators B 97:348
158. Simonis A et al. (2004) Sens Actuators B 103:429
159. Matsuo T, Nakajima H (1984) Sens Actuators 5:293
160. Vandenvlekkert HH et al. (1990) Sens Actuators B 1:395
161. Chovelon JM et al. (1992) Sens Actuators B 8:221
162. Sant W et al. (2003) Sens Actuators B 95:309
163. Oelssner W et al. (2005) Sens Actuators B 105:104
164. Schöning MJ, Brinkmann D, Rolka D, Demuth C, Poghossian A (2005) Sens Actuators B 111:423
165. www.shl-unisensor.com. Accessed 10 Mar 2008
166. www.scilog.com/. Accessed 13 Apr 2008
167. Henning B, Rautenberg J (2006) Ultrasonics 44:E1395
168. Maa YF, Hsu CC (1999) Pharm Dev Technol 4:233
169. Becker T, Mitzscherling M, Delgado A (2002) Food Control 13:223
170. Khanal SK, Grewell D, Sung S, Hans van Leeuwen J (2007) Crit Rev Environ Sci Technol 37:277
171. Schmidt P, Rosenfeld E, Millner R, Czerner R, Schellenberger A (1987) Biotechnol Bioeng 30:928
172. Mohammad A, Khan AG, Kuek C (2000) Mycorhizza 9:337
173. Hauptmann P, Hoppe N, Püttmer A (2002) Meas Sci Technol 13:R73
174. Rama Rao M (1941) Nature 147:268
175. Püttmer A, Hoppe N, Henning B, Hauptmann P (1999) Sens Actuators A 76:122
176. Schneditz D, Kenner T, Heimel H, Stabinger H (1989) J Acoust Soc Am 86:2073
177. Brown LF, Mason JL (1996) IEEE Trans Ultrason Ferroelectr Freq Control 43:560

Disposables in Downstream Processing

Uwe Gottschalk

Abstract Disposable equipment has been used for many years in the downstream processing industry, but mainly for filtration and buffer/media storage. Over the last decade, there has been increasing interest in the use of disposable concepts for chromatography, replacing steel and glass fixed systems with disposable plastic modules that can be discarded once exhausted, fouled or contaminated. These modules save on cleaning and validation costs, and their smaller footprints reduce buffer consumption, water for injection, labor and facility space, contributing to an overall reduction in expenditure that lowers the cost of goods. This chapter examines the practical and economic benefits of disposable modules in downstream processing.

Contents

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Abbreviations

BSA   Bovine serum albumin
cGMP   Current good manufacturing practice
CIP   Cleaning in place
DNA   Deoxyribonucleic acid
LRV   Log reduction value
MuLV   Murine leukemia virus
MVM   Minute virus of mice
PRV   Pseudorabies Virus
SIP   Steaming in place

1   Introduction

The biopharmaceutical manufacturing industry has used stainless steel since its inception, and even 5 years ago it would have been hard to imagine the industry embracing a new concept that stands to make stainless steel redundant. Despite this inevitable inertia from the industry, the tide seems to be turning, and disposable concepts are on the rise in downstream processing [1]. Disposable equipment became popular for upstream production in the 1990s, with the advent of single-use media bags and bioreactors (the subject of most of the first part of this book), and the use of disposable capsules for sterile filtration [2]. Over the next few years, disposable concepts also began to appear in downstream processing. Initially, this was restricted to buffer bags and devices for normal flow filtration, including virus filtration and guard filters for chromatographic columns, but gradually more complex concepts have been introduced, including disposable devices for tangential flow filtration and chromatography [3–6]. The value of disposables in downstream processing is debated, with some expecting a revolution throughout the process chain and others expecting disposables to remain in niche areas [7, 8]. Although the debate continues, there is plenty of information from different biopharmaceutical production campaigns to show that disposables offer real and tangible advantages over fixed stainless steel equipment under many different circumstances. This chapter will consider the issues involved and provide case studies demonstrating the benefits of "going disposable" in biomanufacturing.

2   Why Single-Use Concepts Could Be Beneficial

At first glance, disposables appear wasteful and unnecessary, analogous to taking fresh plastic bags at the supermarket during each shopping trip rather than re-using a sturdier container. However, this analogy breaks down when one considers the constraints under which biomanufacturing processes must labor to ensure that the resulting active pharmaceutical ingredient is safe, pure, homogeneous and suitable for clinical use. The re-usable shopping bag might not turn out to be so convenient if it had to be washed and sterilized before each trip, and if that cleaning had to be validated professionally!

The most commonly cited benefit for the single-use concept is the elimination of potential cross-contamination between production batches or even between batches of different products. Over the last few years the industry has seen a significant shift towards the adoption of disposable bioprocess components because, unlike stainless steel systems, they do not need to be disassembled, steamed, cleaned and reassembled between batches. Instead, components can be supplied as sterile, process-ready modules which are used once and then discarded [1, 4].

In general, the handling of unit operations is simplified by the employment of ready-to-use disposables, and this is probably the only type of disposable unit that provides the full advantage to biopharmaceutical manufacturing operations. The additional costs of replacing disposable components are offset many times over by the cost savings brought about by eliminating cleaning-in-place (CIP) and steaming-in-place (SIP) procedures, validation studies and the associated record keeping. Furthermore, disposable components greatly increase the flexibility of production since they facilitate rapid and inexpensive product changeovers with minimal risk of cross-contamination. Although some of the equipment used for downstream processing still needs to be cleaned (e.g., rotary lobe pumps and mechanical valves), such procedures can be carried out using higher concentrations of chemicals at higher temperatures, therefore significantly reducing process down-time.

Integrated biopharmaceutical fluid-handling steps include media preparation, fermentation, cell harvesting, clarification, product capture and polishing, virus clearance, ultrafiltration and finally sterile filtration. All these unit operations, which formerly relied on stainless steel components, can now be carried out using disposable modules. By switching to a single-use concept, the industry aims to reduce or eliminate the most time-consuming and cost-intensive process steps, ultimately shortening the time to market. Additionally, the regulatory bodies focus on critical production steps such as CIP/SIP [9–11] and these are the very steps that can be abolished by the single-use concept.

3 Economic Considerations

3.1 Overview

The obvious question regarding the use of disposables is how the costs stack up compared to hard-piped components. Although there are obvious savings in up-front investment in equipment, cleaning and validation, is this cancelled out by the greater consumption of consumables, not least the disposable modules themselves? For example, how does the cost of replacing a filter for each process batch compare to the lifetime costs of cleaning and reassembling a permanent filtration device over many process runs?

There is no definitive answer to such generic questions. It is only possible to compare costs for specific process operations, and at specific scales. Below, as an example, I compare the relative costs of column chromatography and disposable membrane adsorbers for polishing in antibody manufacture. As we will see, columns

are more economical at low scales, but costs break even at a load of approximately 2 kg of antibody per liter of resin. At higher scales, single use membrane adsorbers are significantly more economical. Importantly, this reflects not only the cost of equipment, cleaning, validation and consumables, but also the reduced buffer volumes, the reduction in labor, the reduced requirement for water-for-injection, the reduction of process down time, and the higher productivity of the membrane adsorber in this particular setting. The footprint of disposable devices is generally smaller than fixed counterparts with significant knock-on effects in terms of facility layout and design. All the extra buffers required to wash and re-equilibrate fixed equipment need to be stored and prepared somewhere, increasing the overall costs in terms of facility planning and space requirements. Therefore, the use of disposables cannot be evaluated as an unlinked concept, but must be considered in the context of which unit operations are used, their efficiency, scalability and economy. Perhaps one of the most important concepts, often overlooked, is that the use of disposables allows process trains to be assembled rapidly from modules, and scaled up efficiently. A hard-piped process that could take years to finalize can be assembled from disposable modules in a matter of weeks [12].

According to cGMP standards, raw materials and equipment in direct contact with the product need to be dedicated, which makes the most expensive hardware prohibitive for limited production campaigns. Typical hardware might include chromatography columns, filters, filter holders, process control systems, buffer storage tanks, and peripheral utilities such as pumps, valves, piping and monitoring equipment. Disposables can be dedicated not only to a specific product, but to a single batch, and therefore dramatically reduce the initial capital investment in limited campaigns. As well as these fixed costs, savings are likely to be made in terms of reduced lead time for equipment acquisition and qualification, low maintenance and, as stated above, the absence of cleaning requirements. Time is money in biopharmaceutical manufacturing, and clipping 6–12 months off the time taken to develop a final process can reap rewards years downstream by extending the useful life of patents and ensuring that market demands are rapidly fulfilled.

In addition to up-front (fixed) costs, which currently drive the industry, there are also operational costs that can be reduced by using disposables [13]. Such costs include labor, off-line analysis, chemicals and water, chromatography resins and buffers, and the costs of waste treatment and disposal. While disposable options do not eliminate such costs, they can reduce them significantly. A cost comparison is presented later for column chromatography vs disposable membrane adsorbers and the surprising result is that even with the costs of the disposable membranes included, the actual running costs of a disposable production campaign are still lower than those of traditional columns over a 10-year production cycle [13].

3.2 Validation

From a cost perspective the second largest investment after hardware is the cost of qualification and validation in order to make a process available for cGMP

manufacturing. Typically, extensive cleaning validation has to be performed in order to allow the re-use of the equipment for different production batches. In the case of multipurpose use of the equipment for different products, even more extensive studies have to be performed to exclude any potential cross-contamination between products. This is critical for those drugs with significant effects at very low doses, e.g., cytokines [14].

In this respect disposables provide an opportunity to circumvent extensive qualification and validation of the equipment and piping. Single-use components are available for instant use, i.e., they are pre-sanitized and pyrogen-free. The requirement for sterility of single-use equipment depends on the stage of processing, e.g., fermentation or downstream processing or on the type of product to be manufactured. The downstream processing of biopharmaceutical proteins is typically performed in a sanitary, low-bioburden but not sterile environment, whereas the purification of virus vaccines and plasmids may require sterile handling. In any case, suitable disposables eliminate the need for CIP or SIP, and pre-assembled single-use equipment reduces further the potential of operator error and thus contributes to increased process robustness [15]. Accordingly, product change-over in a manufacturing suite is impressively facilitated. The savings in resources – both time and personnel – significantly contribute to reduce turnover time and in this manner allow the installment of additional project(s) without investment in new resources, utilities and facility space. Savings will also be made by reduced analytical costs: as there are no analytical assays for proof of cleaning required, there is no need to develop, qualify, validate and perform individual assays for each new product.

4   The Environmental Impact of Disposables

The seamless logistics of production components is an important factor that contributes to successful and efficient manufacturing. Good process economics requires production to be *lean*, i.e., the correct components must be available on demand, but stocks must be limited to conserve storage space and avoid tying up capital that could be invested in the value stream. A lot of up-front investment is required for stainless steel components, so to a certain extent the flexibility of capital resources is limited when using a hard-piped production system. Similarly, the availability of equipment for CIP and SIP needs to be factored in when looking at the economics of stainless steel. If a CIP station fails, the availability of clean equipment can become critical, particularly if only a limited number of replacements are available.

Single-use components tend to avoid such problems because the demand for different modules can usually be predicted based on the intended production campaigns. It is beneficial to maintain a small surplus stock of filters, membrane adsorbers, media bags etc., but this binds much less capital than analogous replacement stainless steel equipment and would be anticipated as a normal line in the consumables budget. Even where there is critical failure and/or an availability crisis, it is much easier to source disposable equipment and have it shipped to the production facility than would be the case for a steel bioreactor or chromatography column.

One issue raised by the throw-away nature of disposable modules is the impact on the environment, bringing us back to the plastic shopping bag analogy mentioned above. The plastic modules are discarded after each production run and are incinerated, which surely must be much worse for the environment than re-using components hundreds of times? Careful analysis, however, shows that the opposite is true. Because of the demands of working according to cGMP, the cleaning and validation required between batches in a conventional production train is much heavier in its consumption of chemicals, water and energy than the equivalent disposable technology. Although it would be a mistake to regard disposables arrogantly as an environmentally-friendly concept per se, it is by far the friendliest option compared to the traditional approach.

5   Limitations of Disposable Components

Although disposables are beneficial in terms of efficiency, economy and resource management under many circumstances, no one claims they can provide the solution to all manufacturing issues and there are still many situations in which re-usable equipment remains the best choice [16]. Disposable unit operations are available for almost all conceivable unit operations at smaller scales (<50 L harvest reactor volume) and perhaps this is even true for pilot-scale operations. However, at process scale, the cost of manufacturing some types of disposable equipment becomes unsupportable, meaning that the hard-piped alternative is better value, even with the attendant cleaning and validation. In some cases, this simply reflects the relative cost of disposable modules compared to the cost of stainless steel equipment averaged over the larger number of production runs possible before replacement is required. In other cases, the performance of disposable modules fails to match that of fixed equipment at process scale. However, there has been a noticeable trend toward the availability of larger disposable modules, driven by increasing acceptance at smaller scales and the realization that further development and improvement will reap economic rewards in the long term by overcoming process bottlenecks. Process scale chromatography using disposable membrane adsorbers is one example, discussed in more detail below. At the moment, this is applicable to polishing (flow-through mode) but not to capture (retention mode), although it is only a matter of time before this too becomes more economical and efficient using disposable technology [17].

6   Disposables in Downstream Processing

6.1   Initial Recovery

After the fermentation cycle, cell harvesting and clarification is followed by product capture, polishing (including viral clearance) and product filling, all of which have been streamlined through the use of disposable modules. For cell harvesting and

debris removal, disposable filtration systems offer many advantages over their hard-piped counterparts in addition to the general benefits of single-use components listed above. These include the ease of scale up, the availability of pre-sterilized filters which can be integrated directly into production lines, and the fact that abolishing the use of (opaque) stainless steel housings makes it possible to observe the filter in action, and thus identify any potential problems such as foaming or air-locking before the rest of the production line can be affected. The switch from hard-piped steel filters to disposables has been facilitated by the development of disposable filtration systems that use the same cartridges as those used with the stainless steel housings. The increased flexibility afforded by disposables also means that several filters can be arranged in series or in parallel, according to the batch size and the throughput of the bioreactor.

Cell removal and clarification are often achieved by centrifugation and lenticular filtration [18]. The first disposable formats for this technology became available in the form of Millipore's Pod System, which combines two distinct separation technologies in an adsorptive depth filter to enhance filter capacity and retention, while compressing multiple filtration steps into one efficient step. Scale up is achieved by inserting multiple Pods into a holder, with formats allowing 1–5 Pods or 5–30 Pods as required. More recently, Sartorius-Stedim Biotech has produced a multilayer depth filtration system which significantly reduces effluent turbidity while offering improved breakthrough control [19].

Further purification steps focus on bringing the process volume down – an area where crossflow filtration is the technology of choice because the build up of filter cake (the gel layer) on the membrane is slower than is the case for normal-flow filtration devices. Crossflow filters therefore result in extended operating times, but this advantage can be lost if the membranes need to be cleaned regularly. Disposable crossflow filtration cassettes are now widely used in the vaccine industry, where sterile filtration of the final product is not possible. Disposable hardware and consumable components improve safety by preventing cross-contamination, eliminate CIP steps and validation work, reduce the volume of water used during production (because washing and rinsing is no longer necessary), reduce the amount of chemicals used, and improve yield by eliminating the possibility of membrane degradation through long-term use. All these factors help to reduce costs and time to market.

6.2   Chromatography

In traditional chromatography, a steel column is packed with a resin (stationary phase) comprising porous beads made of a polysaccharide, mineral or synthetic matrix conjugated to specific functional groups exploiting different separative principles [20]. A mixture of components in the feed is percolated through the resin, and the differing affinity of feed components for the functional groups of the resin facilitates separation, either by retaining the target and eluting contaminants (retention) or vice versa (flow-through). Column chromatography is the key enabling technology

in all bioseparation processes, but after years of dominance a significant weakness is emerging, i.e., its decreasing performance at process scales [20]. Very large columns remain robust and reliable, but there is no economy of scale with such devices because the additional cost of resins, buffers and other consumables outstrips any savings made by increasing the productivity.

While large columns are required for bind and elute steps – an area that continues to be dominated by Re-usable technology – they are slowly being replaced by membrane-based concepts in flow-through steps for polishing. Here, oversized columns are necessary to accommodate the throughput, which directly impacts facility layouts, costs and infrastructure because the space and buffer volumes for all steps, including preparation and cleaning, also have to be adapted. These are exactly the issues that disposable modules can address. Membrane chromatography employs thin, synthetic, porous membranes that are generally multilayered in a small cartridge, significantly reducing the footprint of the operation. Membranes have equivalent functional groups to corresponding resins and are directly analogous. However, as stated above, they do not need packing, checking, cleaning, re-filling or routine maintenance, and fouled or exhausted modules can be replaced with new ones with minimal process down-time.

Interest in membrane chromatography has grown because they can be used as disposable modules, but there are other advantages in terms of performance that apply to certain chromatography formats. For example, flow-through anion exchange chromatography is used during the purification of monoclonal antibodies to remove high-molecular-weight contaminants such as DNA and viruses. Such molecules do not readily diffuse into the pores of traditional resins (Fig. 1a), resulting in mass transfer resistance and lower efficiency. To ensure that these molecules are retained, greater column bed heights and slower linear flow rates are required to increase residence times. Most polishing steps operate at a flow rate of between 100 and 150 cm h^{-1} and use dramatically oversized columns. In contrast, solutes find their binding sites on membrane adsorbers mainly by convection, while pore diffusion is minimal (Fig. 1b). Because of these hydrodynamic benefits, membrane adsorbers can operate at much greater flow rates than columns, considerably reducing buffer consumption and shortening the overall process time by up to 100-fold [3]. The use of membrane adsorbers can be viewed as the equivalent of shortening traditional columns to near zero length, allowing large scale processes to run with only a small pressure drop at very high flow rates. For example, polishing with an anion exchange membrane can be conducted with a bed height of 4 mm at flow rates of more than 600 cm h^{-1}, but even so the membrane pores provide adequate binding capacity for large biomolecules such as viruses and DNA, so they can play an important role in the overall viral clearance strategy for antibody purification [5, 12].

The importance of flexibility in process assembly has already been discussed, but it is pertinent to focus on scalability. Flexibility is most noticeable during scale-up, since disposable devices are generally modular and available in a number of different sizes, and scaling up simply involves swapping one module for another with a higher capacity. As shown in Table 1, an important advantage of membrane chromatography is the linear scale up for important parameters such as frontal surface area, bed volume, flow rate and static binding capacity, while normalized dynamic capacity
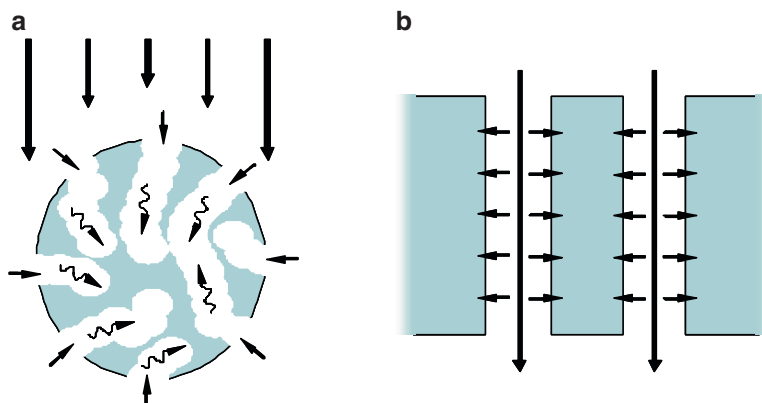
Fig. 1a,b Mechanistic comparison of solute transport in (**a**) packed-bed and (**b**) membrane chromatography. *Thick arrows* represent bulk convection, *thin arrows* represent film diffusion and *curly arrows* represent pore diffusion.

Table 1 Scale up with SingleSep Q membrane chromatography

	Frontal surface area (cm²)	Scale-up factor for flow rate	Rec. flow rate (L min⁻¹)	Bed volume (mL)	Min. static binding capacity (g) (Release test)	Dynamic capacity at 10% (mg mL⁻¹)	Dynamic capacity at 100% (mg mL⁻¹)
Nano	2.4	1	0.03	1	0.03	22.5	39
5	160	66	1.9	70	2.0	19.5	30
10	450	187	5.0	180	5.3	20.5	29.5
20	900	375	10	360	10.5	20.5	35
30	1,350	562	15	540	15.8	20.5	37.5
Mega	4,050	1687	45	1,620	47		

Parameters such as frontal surface area, bed volume, flow rate and static binding capacity scale up in a linear fashion (assuming constant bed height of 4 mm). Normalized dynamic BSA binding capacity remains constant at a given breakthrough (values shown at 10% and 100%; see also Fig. 2). Data from Sartorius-Stedim Biotech.

remains fairly constant at 10% or complete breakthrough (Fig. 2). It is thus apparent that membrane devices can be scaled up with none of the attendant disadvantages of column resins.

7 Economic and Performance Case Study

The first membrane adsorbers suffered from problems related to both adsorptive capacity and device performance, e.g., low loading capacity, membrane fouling and suboptimal fluid distribution leading to a substantial performance loss during scale-up [21]. However, these issues have been largely addressed by the development of
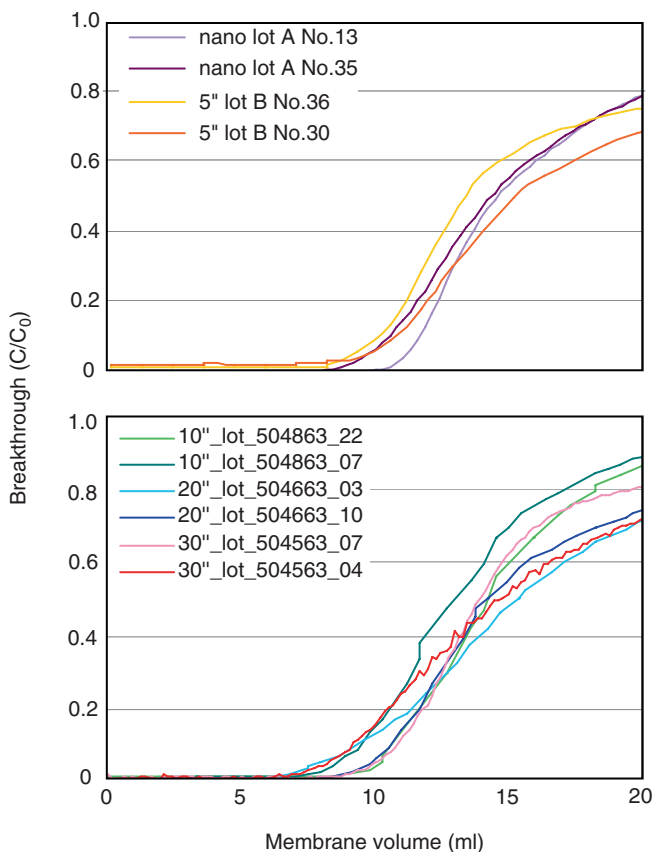
Fig. 2 Dynamic binding capacities of SingleSep Q membrane chromatography devices represented by breakthrough values as percentage of total load (C/C_0) against membrane volume (mL). Individual curves represent selected lots of different sized devices ranging from nano to 30″.

improved surface chemistries and the design of membrane devices that optimize performance. In process scale operations, 15-layer devices are commonly deployed and these achieve excellent contaminant removal and viral clearance results. For example, a flow through membrane chromatography case study designed to reflect process scale conditions and performed with a 3.5-mL/125-cm² spiral wound scale down device achieved log reduction values (LRVs) of >5 for four model viruses (Table 2). These performance studies confirm that both columns and disposable membrane adsorbers are capable of trace contaminant removal and virus clearance. The main difference between the two formats is disposability and load capacity at flow rates acceptable for large-scale manufacturing. The process capacity of multi-layer Q membranes is much higher than equivalent volumes of resin with no loss of performance in contaminant and virus removal. With performance assured, the remaining question is how disposable membrane devices compare columns in terms of cost

Table 2 Membrane chromatography spiking study with four model viruses

Virus	Size (nm)	LRV (run 1)	LRV (run 2)	Virus recovery (%)
MVM	16–25	6.03 ± 0.21	6.03 ± 0.20	100
Reo-3	75–80	7.00 ± 0.31	6.94 ± 0.24	100
MuLV	80–110	5.35 ± 0.23	5.52 ± 0.27	>70
PRV	150–250	5.58 ± 0.28	5.58 ± 0.22	100

Data from [22]

Test substance was a human monoclonal antibody (5–9 g L^{-1}), pH 7.2; 4 mS cm^{-1}; 1% spike; 450–600 cm h^{-1}.

– both fixed (capital) costs and variable (running) costs. Capacity and disposability are critical factors to consider when calculating unit operation costs for new processes. Although membrane devices clearly have a higher throughput, a direct comparison of resins and membranes based on volume shows that membranes are more expensive. This must be balanced, however, against the reduced size of membrane devices, which also reduces buffer requirements, makes the process time shorter and comes along with all the other benefits from a disposable technology [13].

A 10-year cost model [23] showed that Q-membrane chromatography was economically unfeasible compared to Q-resin columns at a process capacity of 500 g m^{-2} (equivalent to about 1.8 kg L^{-1}) mainly due to the cost of membrane devices. The model was based on an upstream CHO platform featuring a 15,000-L bioreactor with a yield of 1 g L^{-1} antibody. This generates a load of 13–15 kg of antibody per batch, which would require a 220-L column or a 1.6-L membrane device based on typical performance standards. The model assumed that up to 40 batches could be run in a year, with the column resin replaced after each 100 cycles. Therefore, the column would need to be repacked with resin four times during the process lifetime, whereas 400 membrane devices would be required over the same period. The model suggested that capacity would need to increase above 2 kg m^{-2} (7.2 kg L^{-1}) to become competitive.

Another cost model [24, 25] suggests that membrane chromatography could break even with resins at a load of just 2 kg L^{-1}. With a load capacity of 10 kg L^{-1}, the membrane-based process costs only one-fifth as much as an equivalent operation using resins (Fig. 3). This cost of goods model was based on the use of 10-in. Sartobind SingleSep Q ion-exchange capsules (180 mL volume). Costs were based on a 10-year process lifetime with column resins replaced after 100 cycles, although in this case the output was from a 10,000-L bioreactor. The values of 10 kg L^{-1} and 2 kg L^{-1} were considered typical for a relatively pure (late stage) feed stream after intermediate polishing and a less pure (earlier stage) feed stream, after clarification and capture by Protein A chromatography. The unique aspect of the model was its consideration and separation of all direct and indirect costs into four major categories – capital equipment, consumable equipment and media, consumable chemicals and materials, and labor. As might be expected, the fixed capital cost was the most significant in the case of column chromatography whereas the cost of consumable equipment and media was higher for membrane chromatography because the mem-
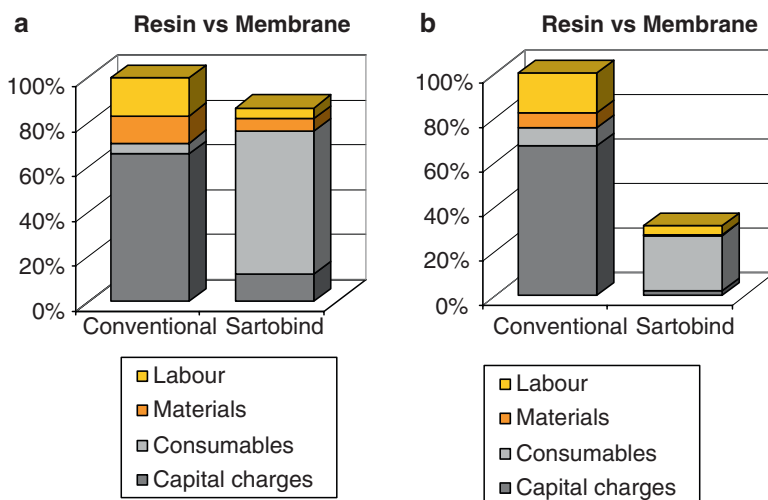
Fig. 3a,b Comparative results from a cost model comparing traditional and membrane chromatography [18, 19], showing each component (labor, materials, consumables and capital charges) as a percentage of the total cost of column chromatography (which is fixed arbitrarily at 100% so that the savings brought about by membrane chromatography can be shown as a percentage cost reduction per batch). Costs break even at a load capacity of 2 kg L^{-1} (**a**) and at 10 kg L^{-1} (**b**) membranes cost only 20% as much per batch as running a column.

brane device needs to be replaced after each batch while the column resin can be cleaned and regenerated and the costs therefore spread over 100 cycles. However, the consumption of membrane is lower at the higher loading capacity since fewer capsules need to be used. The use of other consumables is much higher in the case of column chromatography because the resin needs to be washed and regenerated, and the large size of the column demands higher volumes, and the cost of labor is approximately fourfold higher because of buffer preparation, cleaning, validation, maintenance of equipment and quality work.

8   Conclusions

Single use and disposable equipment is widely accepted and well-established in biopharmaceutical processing at scales of up to several thousand liters. The application of this concept can save a significant investment in hardware, if the intention is limited use for individual applications, e.g., a limited number of process batches. However, as shown by the example of disposable membrane chromatography in antibody polishing, it can also provide savings throughout the lifetime of a production campaign by providing better economy of scale than traditional approaches. Single use equipment is attractive for cGMP applications, as it is provided ready to use. Thus it can save valuable labor

costs, particularly in terms of cleaning and validation. Single use equipment may help significantly to reduce the turnover time between campaigns. Additional projects can be implemented instead, thus increasing the profitability of the facility. However, the use of disposable concepts should always be considered on a case-by-case basis, taking into account individual properties of the manufacturing facility, its infrastructure and the technical details of all processes.

References

1. Sinclair A, Monge M (2004) BioProcess Int 2:26
2. Meyeroltmanns F, Schmitz J, Nazlee M (2005) BioProcess Int 3:60
3. Walter JK (1998) In: Subramanian G (ed.) Bioseparation and bioprocessing. processing, quality and characterization, economics, safety and hygiene. Wiley-VCH, p 447
4. Ransohoff T (2004) Poster Presentation at BPD North Carolina Biotechnology Center
5. Gottschalk U (2006) Biopharm Int 19(Suppl):8
6. Muller-Spath T, Morbidelli M (2009) In: Gottschalk U (ed.) Downstream processing of antibodies. Wiley, New York (in press)
7. Kelley B (2007) Biotechnology Prog 23:995
8. Low D, O'Leary R, Pujar NS (2007) J Chromatogr B 848:48
9. European Commission Enterprise Directorate General (2001) Working party on control of medicines and inspections. EU guide to good manufacturing practice, vol. 4, Annex 15, July 2001, Cleaning Validation
10. FDA (2004) Equipment cleaning and maintenance. Code of Federal Regulations (CFR) Part 211.67 Title 21 Rev. FDA, Rockville MD
11. FDA (2006) Guide to inspections validation of cleaning processes. FDA, Rockville MD
12. Gottschalk U (2005) BioPharm Int 18:24
13. Mora J, Sinclair A, Delmdahl N, Gottschalk U (2006) Bioprocess Int 4(Suppl):38
14. CPMP (1996) Note for guidance on virus validation studies. The design, continuation and interpretation of studies validating the inactivation and removal of viruses. CPMP/BWP/268/95
15. Immelmann A, Kellings K, Stamm O, Tarrach K (2005) Bioprocess Int 3:38
16. DiBlasi K, Jornitz MW, Gottschalk U, Priebe PM (2006) BioPharm Int (suppl) 19:6
17. Gottschalk U (2008) Biotechnol Prog 24:496
18. Prashad M, Tarrach K (2006) FISE 9:28
19. Tarrach K, Köhler K, Grimm C (2008) Pharma Tech Eur 20:1
20. Curling J, Gottschalk U (2007) BioPharm Intl 20:70
21. Gebauer K, Thommes J, Kula M (1997) Biotechnol Bioeng 54:181
22. Gottschalk U, Lamproye A, Zhou J, Sinclair A, Reif O-W (2006), Poster Presentation at Recovery of Biological Products XII
23. Zhou J (2006) IBC Technology Transfer for Biopharmaceuticals
24. Sinclair A, Monge M (2002) Pharma Eng 22:20
25. Lim JAC, Sinclair A, Kim DS, Gottschalk U (2007) Poster Presentation at BioProcess Intl 5:60

Hybrid and Disposable Facilities for Manufacturing of Biopharmaceuticals: Pros and Cons

Aline Ravisé, Emmanuelle Cameau, Georges De Abreu, and Alain Pralong

Abstract Modern biotechnology has grown over the last 35 years to a maturing industry producing and delivering high-value biopharmaceuticals that yield important medical and economical benefits. The constantly increasing need for biopharmaceuticals and significant costs related to time-consuming R&D work makes this industry risky and highly competitive. This trend is confirmed by the important number of biopharmaceuticals that are actually under development at all stages by all major pharmaceutical industry companies. A consequence of this evolution is an increasing need for development and manufacturing capacity. The build up of traditional – stainless steel – technology is complicated, time consuming and very expensive. The decision for such a major investment needs to be taken early in the development cycle of a promising drug to cope with future demands for clinical trials and product launch. Possibilities for the reduction of R&D and manufacturing costs are therefore of significant interest in order to be competitive.

In this chapter, four case studies are presented which outline ways to reduce significantly R&D and manufacturing costs by using disposable technology in the frame of a the transfer of an antibody manufacturing process, the preparation of media and buffers in commercial manufacturing and a direct comparison of a traditional and a fully disposable pilot plant.

Keywords disposable bioreactors, disposable faciltities, hybrid facilities, manufacturing of biopharmaceuticals

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Contents

1   Introduction

Modern biotechnology founded on the principles of recombinant DNA (rDNA) protein production began in the early 1970s with the discovery and development of molecular biology and genetic engineering techniques to manipulate DNA and transform cells [1]. Over the last 35 years the biotechnology industry has grown continuously to a maturing industry and human medicine and health care is the most prominent field of application. Biopharmaceuticals produced by modern biotechnology yield important benefits such as unique therapeutic and diagnostic solutions, unlimited supplies of potentially safer products as well as superior therapeutic and diagnostic approaches, respectively [2].

Biopharmaceuticals are relatively high-value products and the annual sales of approved biopharmaceuticals were estimated at \$33 billion back in 2005 [3]. The expectations of sales values reached for therapeutic monoclonal antibodies (mAbs) by 2008 are \$16.7 billion [4], and the revenues forecasted from non-mAb-based therapeutic proteins are \$52 billion by 2010 [1]. Summarizing, it is forecasted that the total biopharmaceutical market should approach or perhaps exceed \$70 billion by the end of 2010 [5].

These projections led in the past to major investments in R&D activities for biopharmaceuticals and biotech-based products are increasingly dominating the
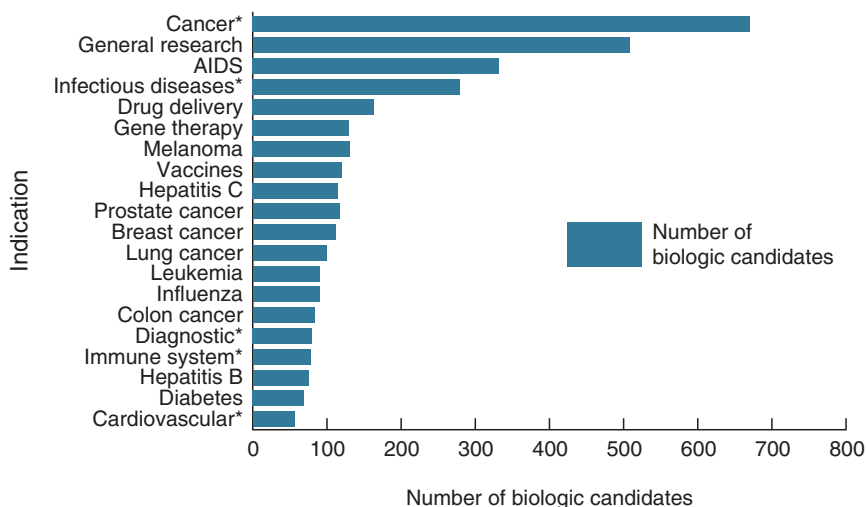
Fig. 1 Repartition of biotech drugs in development per target indication. Cancer is the most prominent target indication for biopharmaceuticals, whereas mAbs and vaccines represent the most significant categories by product number [5]

pipeline of biopharmaceutical companies. Back in 2006, it was estimated that 2,500 biotech drugs were in the discovery phase, 900 in pre-clinical trials and over 1,600 in clinical trials [5]. In Fig. 1 is shown the repartition of biotech drugs in development per target indication. The annual biopharmaceutical R&D expenditure was estimated to $19–$20 billion from 2004 to 2006 [4].

In view of the total biotech pipeline, availability of manufacturing capacity to support clinical development and market production is key for the success of this industry. In 2005, the total global manufacturing capacity was estimated at 475,000 L and continued to increase [6]. In between, larger biopharmaceutical companies have bioreactor capacities up to 25,000 L for mammalian fermentation. The build up of manufacturing capacity is a lengthy task that takes 5–8 years for a complete plant and signifies major investments by the company.

During R&D activities, the risky decision to build up manufacturing capacity has to be taken early in the development of a new promising drug in order be able to cope with the Active Pharmaceutical Ingredient (API) requirements for clinical trials and the potential future market demand. These constraints result in a significant increase of costs for R&D activities and risk in this highly competitive environment.

Furthermore, mounting health care costs are a challenge for many health care systems. Application of modern biotechnology could contribute to reducing health care cost but, on the other hand, the significant investments to develop the biopharmaceutical and provide state of the art manufacturing capacity make biopharmaceuticals expensive drugs [2].

The rising pressure to reduce costs for R&D activities, manufacturing and acceleration of drug development time with the concomitant reduction of the overall investments led over the last 5 years to significant developments in disposable technology. The application of disposables offers various advantages over traditional stainless steel facilities that are outlined in this chapter.

Four case studies are presented, exhibiting actual and possible applications of disposable technologies. In case study I, Alain Pralong outlines the application of disposable technologies in the frame of the transfer of an antibody manufacturing process between two sites. Aline Ravisé and Georges De Abreu present the preparation of fermentation media and purification buffers using disposables in case studies II and III. A traditional stainless steel pilot plant for mammalian fermentation is compared by Emmanuelle Cameau with a fully disposable 2,000-L pilot plant in case study IV.

2 Case Study I: Technology Transfer of a Registered Antibody Manufacturing Process at Roche, Switzerland

2.1 Abstract

A registered antibody process is transferred to a new manufacturing facility. To reduce the overall project time, construction and process transfer were initiated concomitantly using an existing 25-year-old microbial fermentation pilot plant for the interim implementation of a scale down process. This plant needed major adaptations for mammalian fermentation within a very short lead-time. Disposable technology was widely applied to refurbish and operate the plant and maintain the timing.

2.2 Frame of Case Study

This study describes and outlines the extensive use of disposable technology during a technology transfer of a registered antibody manufacturing process from a US-based manufacturing site (donor site) to a new manufacturing site (receiving site) in Europe.

In order to reduce the overall project time consisting of the facility construction, the transfer of the new manufacturing process and the registration of the manufacturing site, it was decided to split the technology transfer into two consecutive steps to allow activities of facility completion and technology transfer to occur in parallel. This strategy could only be adopted as a 25-year-old facility for the cultivation of microorganisms already existed at the receiving site that served as pilot plant.

The first step was initiated while the construction was still ongoing and consisted of transferring the process from the donor site to the existing pilot plant at the receiving site following a scale down approach. The second step consisted of transferring the process from the pilot plant to the new manufacturing facility at the receiving site following an internal scale up approach.

This two-step transfer strategy offered the possibility to gather experience with the process, train personnel and setup procedures for infrastructure operations and purchase of raw materials while the new facility was still under construction. This resulted in a significant gain of time.

The major challenge in following this strategy was to upgrade the existing pilot plant, originally designed for microbial fermentation, to a state where a mammalian cell culture process could be run. The modifications had to be performed in a limited

time frame of about six months. The facility had an outdated technology that had to be adapted to mammalian cell technology. The facility consisted of fermentation and a purification area. The fermentation area was equipped with bioreactors of different sizes (10-L/30-L/100-L/1,000-L) and the downstream area was designed to purify as much as 1,000-L of harvested cell culture fluid. In Fig. 2 are shown
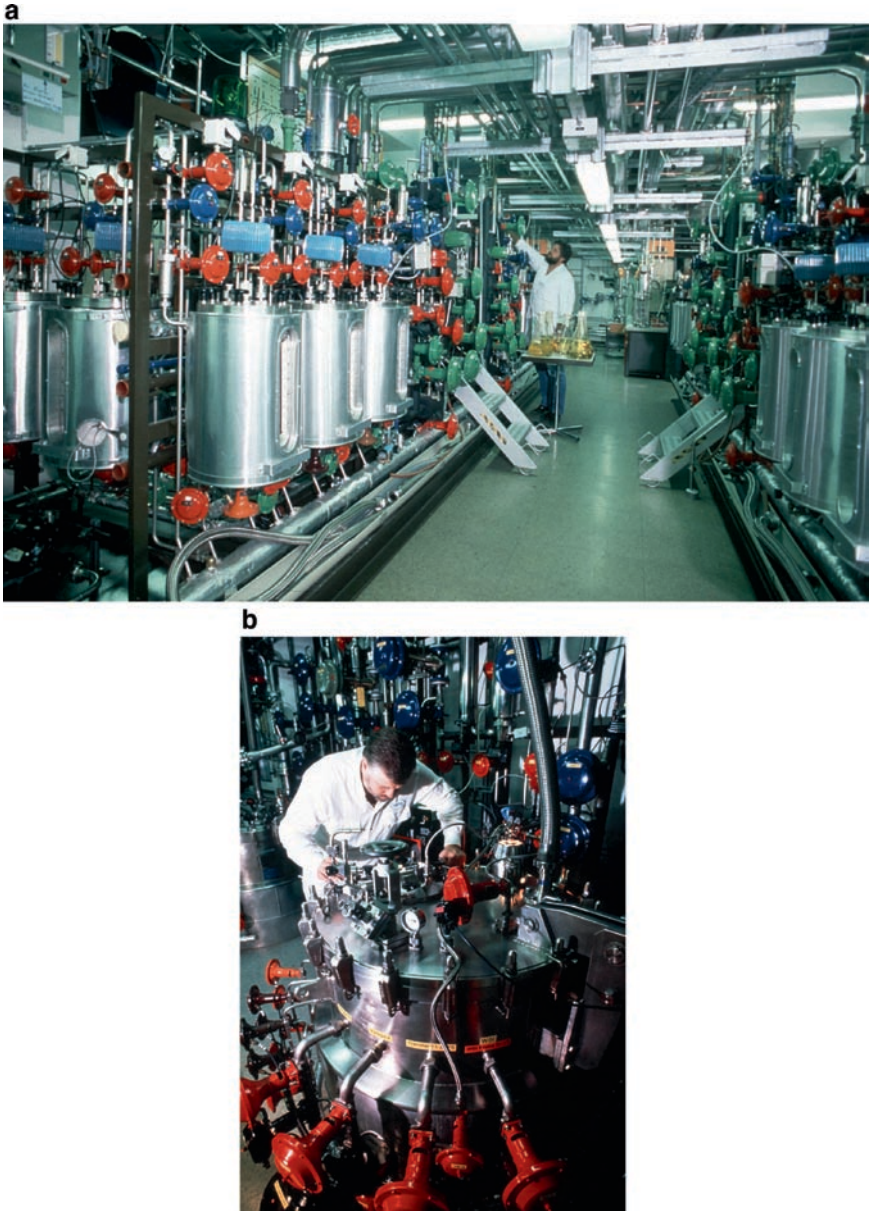
Fig. 2 a Rack consisting of two 100-L bioreactors, control units and vessels for feed, ph-control and antifoam solutions. **b** Head of a 25-year old 100-L bioreactor

installations of the existing fermentation suite. While the existing purification equipment could be easily adapted to the recovery of a monoclonal antibody, major adaptations were necessary in the fermentation area. Limited space as well as tight timelines required structural measures to be reduced to the minimum. Therefore, disposable technology was found to offer straightforward solutions in three functional areas, namely media preparation, cell culture and harvest [7–10].

2.3　Process Requirements and Description

The antibody manufacturing process transferred to the receiving site had several requirements regarding media preparation and cell culture strategy. The cell culture process (1) requires more than five different types of media and (2) is at true scale cultivated over a wide range of bioreactor scales.

The manufacturing process is initiated with the thawing of an ampoule. This culture is expanded using traditional techniques until a seed train bioreactor (20-L) can be seeded. The seed train bioreactor is repeatedly sub-cultivated by removing one part of the cell suspension and diluting the remaining cells with fresh, pre-warmed media to reach the inoculation cell density range. This procedure, called Solera, allows maintaining of the cell source and providing cells as required by manufacturing until reaching the maximum cell age limit. For each API-batch, an inoculum fermenter train consisting of three bioreactors of increasing volume (80-L, 400-L and 2,000-L, respectively) is initiated through inoculation with cells from the seed train bioreactor. The cells are sequentially expanded by exponential growth. The cell culture fluid of the 2,000-L bioreactor is finally used to inoculate the production bioreactor (12,000-L), which is then operated in fed-batch mode. In Fig. 3 is shown a process flow chart of the transferred antibody manufacturing process at true scale.

In order to implement the antibody manufacturing process in the pilot plant, a scale down of the original process and an adaptation to limited bioreactor number and sizes available was necessary. It was decided to perform more than one cell culture stage in the same bioreactor. This meant that individual steps of the inoculum train and the production step of the antibody manufacturing process were mimicked in the scale-down model by consecutive Solera operations on the same bioreactor. After removal of one part of the cell suspension, the remaining cells were diluted with fresh, pre-warmed media used in the next process step to reach the inoculation cell density range.

A 30-L bioreactor was used as seed train bioreactor and repeatedly sub-cultivated to maintain the cell source and a 100-L bioreactor to reproduce the inoculum train and the production step. This strategy allowed furthermore running the pilot plant at its highest capacity. In Fig. 4 is shown a process flow chart of the antibody manufacturing process implemented in the pilot plant.
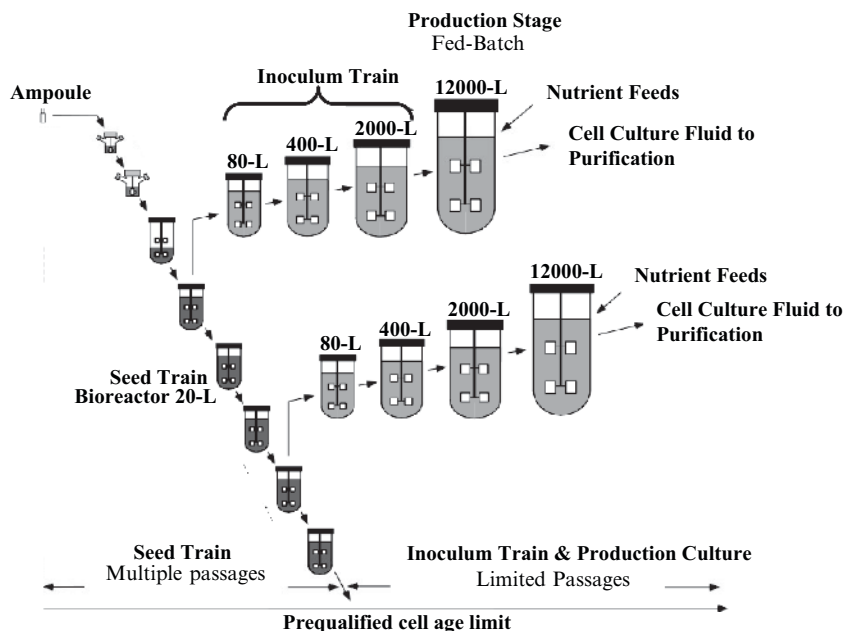
Fig. 3 Upstream production steps of the transferred antibody manufacturing process at true scale. An ampoule is thawed and expanded to inoculate a seed train bioreactor. This bioreactor is repeatedly sub-cultivated to maintain the cell source to initiate an API-batch through inoculation of an Inoculum Train bioreactor used to expand the cells prior inoculation of a production bioreactor

2.4   Media Preparation

As mentioned earlier, the pilot plant was previously used for microbial fermentations and media was directly prepared in the bioreactor. Media and the bioreactor were sterilized prior to inoculation in a single step. In order to implement the antibody manufacturing process, a media preparation facility had to be designed and implemented into the existing facility.

The requirements for the scale down process implied that all types of media could be prepared in a range of 30-L to 500-L scale and that the media aliquots could be pre-warmed prior addition to a bioreactor up to a volume of 100-L. Furthermore, media preparation operations should be well coordinated with the production schedule in order to limit the total number of manifolds used for the transfer into the pilot plant as sterilized and dispensed media could be stored up to three months in a cold room.

2.4.1   Media Preparation Strategy

Media up to volumes of 60 L per batch were prepared in dedicated plastic containers. Mixing was performed using an external stirrer and these plastic containers were
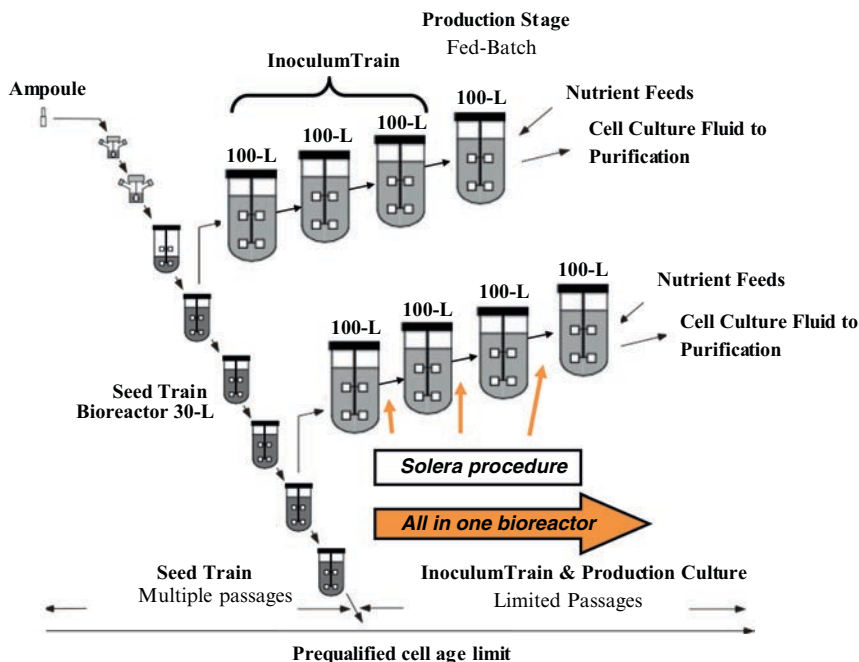
Fig. 4 Upstream production steps of the antibody manufacturing process implemented in the pilot plan after its scale down and adaptation to the existing equipment. All steps of an API-batch production consisting of three inoculum train steps and the production step were performed in the same bioreactor. Transfers of cell culture fluid between bioreactors were mimicked through Solera operations

thoroughly cleaned after each use. In the event of a change over to another manufacturing process, the strategy was to eliminate these containers.

Media from 200 to 500-L batch scale were prepared in customized disposable bags placed in steel bag holding vessels. These bags were equipped with 2.5 cm diameter-tubing for mixing through recirculation and media components such as powders and solutions were added through a spout opening on the top of the bag. In addition to the recirculation loop, the mixing bags had two bottom drain tubings accessible through openings in the steel vessel. These tubings were used for (1) addition of water and (2) draining the media bag for dispensing and filter-sterilization of the prepared media. The steel vessel holding the bag stood on load cells that allowed the monitoring of the amount of media prepared or batched.

2.4.2 Dispensing and Bioburden Reduction of Media

Finished media were non-sterile after preparation and were dispensed into disposable sterile manifolds consisting of multiple storage bags, sampling bags and connection devices connected through tubing and various fittings. Sterilization of the media was achieved through 0.1-mm filters integrated into the manifolds. These

filters served as sterility barrier after gamma sterilization by the supplier. Manifolds were designed following a ready-to-use approach. A 0.2-mm filter prepared in house was inserted after the mixing bag or mixing container to protect the 0.1-mm filters from clogging. Figure 5 shows an example of such a manifold.

The media was pumped from the mixing bag or mixing container through the filters into the storage and sampling bags, which were disconnected after filling from the manifold by sealing off using a thermoplastic tube sealing device. This procedure allowed preparation and dispensing of media without the need of open manipulations in a laminar flow hood. In Fig. 6 a picture sequence for the media preparation and dispensing is shown.

2.4.3 Adaptation of the Facility

As outlined earlier, the pilot plant was not equipped for media preparation. In order to comply with the short project timelines and to reduce time and investments for facility transformation, the concept of a disposable media preparation was adopted. Only mobile bag holding vessels (200-L/500-L) and heating-cooling systems were installed in order to reduce the modification of the facility to the electricity and water supplies. These bag holding vessels (200-L/500-L) used to prepare and store media were jacketed to allow temperature-control. The 500-L unit had an additional
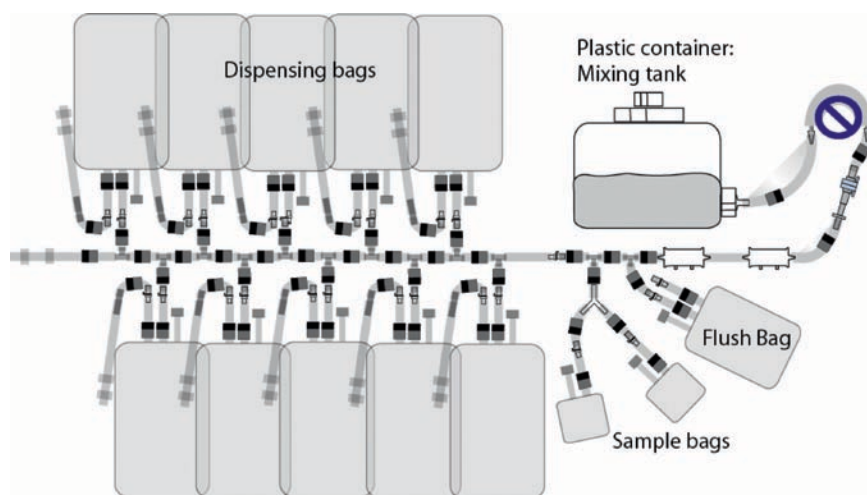
Fig. 5 Example of a manifold used to dispense medium from a plastic container. To the outlet of the container are connected a 0.2- and a 0.1-mm filter. The 0.2-mm filter is prepared in house and connected in between the container and the Gamma sterilized manifold. The 0.1-mm filter is the sterility barrier of the manifold. The manifold consists of a flush bag for the filters, two QC sampling bags and ten storage bags. The bags are disconnected from the manifold individually using a thermoplastic tube sealing device

Fig. 6.a Preparation of 500-L media. Mixing station (1) and the 0.1-mm filter for bioburden control of the added WFI water used for media preparation. *In the back* is the mobile temperature control unit of the mixing station. Media preparation manifold (2) placed inside the mixing station. The water volume added is controlled by weight. Spout (3,4) used for the addition of the media components. This spout is closed during mixing through recirculation using peristaltic pumps (5). The volume is controlled through weight during media preparation. **b** Various steps for media dispensing after preparation. The 500-L mixing station (1) with the prepared media. The peristaltic pump (2) used to dispense the media from the preparation bag to the storage bag, the 0.2- and 0.1-mm filters, the flush bag for the filters and the quality control (QC) sampling bags, respectively. Empty storage bag (3,4) placed in a steel vessel that is itself located on a jack lift with an integrated scale for weight control. Flush bag (5,6) for the filters and the QC sampling bags, respectively

steel frame mounted onto the base of the bag holding vessel that held the pumps and the weight terminal. The weight of the 200-L unit was measured using a jack lift. The heating-cooling system allowed media to be prepared at temperatures higher than ambient to dissolve some of the components more rapidly and cool

down media after dispensing in order to control and reduce cooling time. Media in 100-L bags were pre-warmed in the jacketed holding vessels before addition to a bioreactor. Smaller media volumes were pre-warmed in an incubator.

2.4.4   Drawbacks

The outlined strategy had two major limitations. First, media preparation using mobile equipment was limited to volumes up to 500 L per batch since operators without help of engine driven jack lifts could not safely handle larger volumes. Second, mixing through recirculation may not be as efficient as traditional mixing using an impeller, which may result in increased mixing times. Also, design and review of the manifolds was very time consuming since different types of media and volumes required various types of filters combined with various sizes of bags.

2.4.5   Benefits

Using the strategy outlined above, a fully disposable media preparation for more than five different media compositions at various scales was established in only a few months. The introduction of gamma sterilized and ready-to-use manifolds reduced the workload tremendously since preparation and cleaning of vessels (larger than 60-L) were not required anymore. Furthermore, manifolds were designed to minimize the number of manipulations performed by personnel following a ready-to-use "plug'n play" philosophy (see Fig. 5), which resulted in a negligible number of contaminations.

2.5   Cell Culture

In cell culture, disposable pieces of equipment were implemented in direct contact with the stainless steel bioreactor for addition of media and solutions, sampling and transfer of cells between bioreactors, as well as harvest.

2.5.1   Addition of Media and Solutions

To add media and solutions for pH-control, feed and antifoam were applied to bioreactors in two different ways. In the first option, each storage bag of the pre-sterilized, ready-to-use manifolds was equipped with a disposable three-way valve connector (Steam-Thru-Connector®) that was mounted onto the bioreactor using Tri-clamp fittings (see Fig. 7). This connection was steam sterilized prior to use. After cooling down, the connector was moved from the sterilization to the pre-sterilized open position to transfer media or solution into the bioreactor.

The second option for adding media and solutions was to establish a connection to the bioreactor using thermoplastic tube welding. This technology however
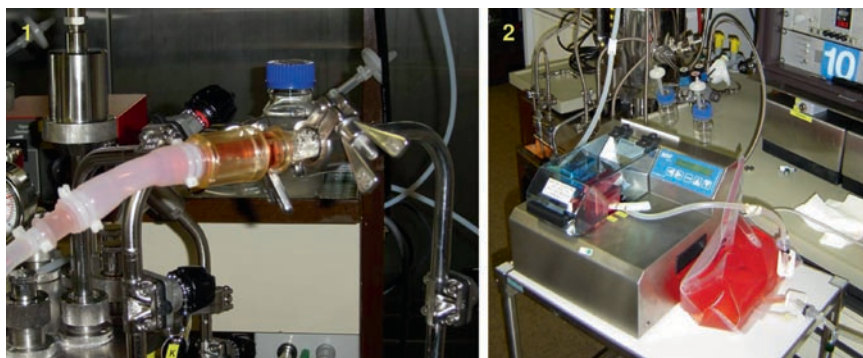
Fig. 7 Two ways used to perform connection to bioreactors. Steam-Thru-Connector® (*left*) mounted on a 10-L bioreactor. This connection is steam-sterilized prior to its use. Tube-welding device (*right*) used for the rapid connection of sensitive solutions such as cell suspensions

needed the preparation of thermoplastic tubing that was separately autoclaved and then mounted on the bioreactor to prepare it for the future welding operation. The storage bags of the manifolds were pre-equipped with weldable tubing. Generally, bags were disconnected from manifolds and connection to bioreactors using a thermoplastic tube sealing device.

The volume of media or solution added or removed was controlled by weight based either on the bioreactor load cells or by putting the bag on a scale. The second option was of great value as the load cells of the old bioreactors could be inaccurate. For larger volumes (100 L), bags were kept in bag holding vessels (see Fig. 1) and weight was controlled using a scale integrated in the jack lift.

2.5.2 Transfer of Cells

Transfer of cells between bioreactors (30- to 100-L) or between spinner vessels (500-mL/1-L/3-L/8-L) and bioreactors (30-L) were made using special manifolds prepared at the pilot plant that were equipped for thermoplastic tube welding. A gamma sterilized transfer bag was equipped inside a laminar flow hood with autoclaved thermoplastic tubing. This manifold was connected to and disconnected from bioreactors or a spinner vessel using thermoplastic tube welding and sealing, respectively.

The amount of cell culture fluid to be removed or added was determined based on the result of a viable cell density count. The volume corresponding to this calculated amount was controlled by weight using scales and the load cells of the bioreactor.

For the transfer of cells, the welding method was preferred to using disposable one or three-way valve connectors, as no steam sterilization and cooling were required. The time used for these operations would expose the cells inside the bag

to uncontrolled conditions during approximately one hour as far as pH, oxygenation and temperature was concerned.

2.5.3   Solera Operation

To perform a Solera operation, the volume of cell culture fluid to remove was determined as explained earlier and controlled by weight. For the Solera operation performed on the 30-L bioreactor, the bag was placed on a scale. The bag used for the Solera operation on a 100-L bioreactor was placed in a bag holding vessel and the weight controlled by a scale integrated into a jack lift. Control of media volume added was performed following the same approach.

2.5.4   Sampling

The sampling of bioreactors was performed using a gamma sterilized, ready-to-use manifold consisting of two sampling bags connected to a one-way valve connector (Lynx®) through thermoplastic tubing (see Fig. 8). The manifold was mounted onto the bioreactor outlet and fixed using a Tri-clamp fitting. To isolate a sample,
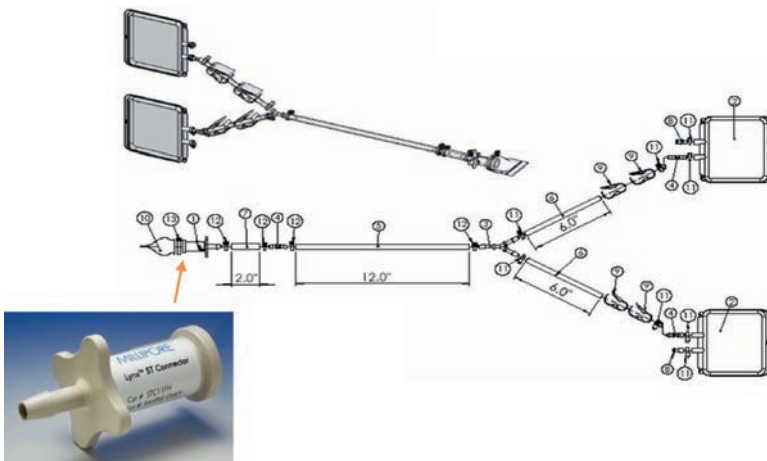
Fig. 8 Manifold used for the sampling of bioreactors. The manifold consists of two 50-mL bags that are connected through thermoplastic tubing to a Lynx® connector. The manifold can be connected through the Lynx® to the bioreactor via a mini Tri-clamp on the sampling line. This line and the contact surface of the Lynx® can be steam-sterilized before sampling. By twisting the Lynx® connector it can be opened and the samples isolated. By twisting the Lynx® back into the original position the sampling line can be closed again and cleaned through steam-sterilization. The sample bags are disconnected from the manifold using a thermoplastic tube sealing device

the bioreactor outlet valve was steam-sterilized together with the outside of the connector and cooled down. Then, the one-way valve connector was opened by twisting prior to bioreactor sampling and closed after transfer of fluid into the sample bags. A sterilization cycle of the bioreactor outlet valve was initiated to clean the bioreactor outlet and reduce the contamination risk. The sample bags were disconnected from the manifold through thermoplastic tube sealing. The sample bags were furthermore equipped with Luer-Locks to allow sterile retrieval of samples for analysis or sterility tests.

The outlined sampling procedure using a ready-to-use manifold reduced the workload and contamination risk of this procedure considerably by avoiding cleaning, preparing and autoclaving of traditional glass bottles.

2.5.5 Harvest

The harvest of antibody-containing supernatant at the true scale manufacturing process was performed using a centrifugation step followed by a filter cascade consisting of depth and bioburden reduction filters. In the pilot plant, the harvest of the 100-L bioreactor was performed using disposable depth filters to remove cells followed by a scale down of the depth and bioburden reduction filter cascade to clarify the supernatant (see Fig. 9). The supernatant was collected into a disposable sterile manifold using methods described earlier and transferred to the purification area.

2.6 Conclusion

The solutions presented for the upstream part of a mammalian cell culture process show that disposable technology has reached today the level necessary to provide the tools needed to prepare media, operate bioreactors and isolate antibody-containing supernatant. The choice to work with disposable tools had a tremendous impact on the success of the whole process transfer as the flexibility of combination of the different disposable tools and the potential for customization allowed meeting the requirements of the process and an already designed facility (retrofit).

All solutions were developed within 6 months while the facility was in use for another project. The design of the solutions was focused on reducing the workload and the number of critical operations such as sterile connections in order to reduce the failure rate. This disposable strategy proved to be successful as shown by the high success rate of the cell culture runs (97%, $N = 271$) supporting a rapid implementation of the manufacturing process in the pilot plant. These results were further confirmed with cell culture and product quality data gathered from the pilot plant operations.

Minimizing costs was not the primary goal for the disposable approach, although in the end only minor investments in hardware and facility refurbishment were necessary for the retrofit. As a consequence of the significant reduction of workload for the preparation of the different operations and the cleaning, no additional
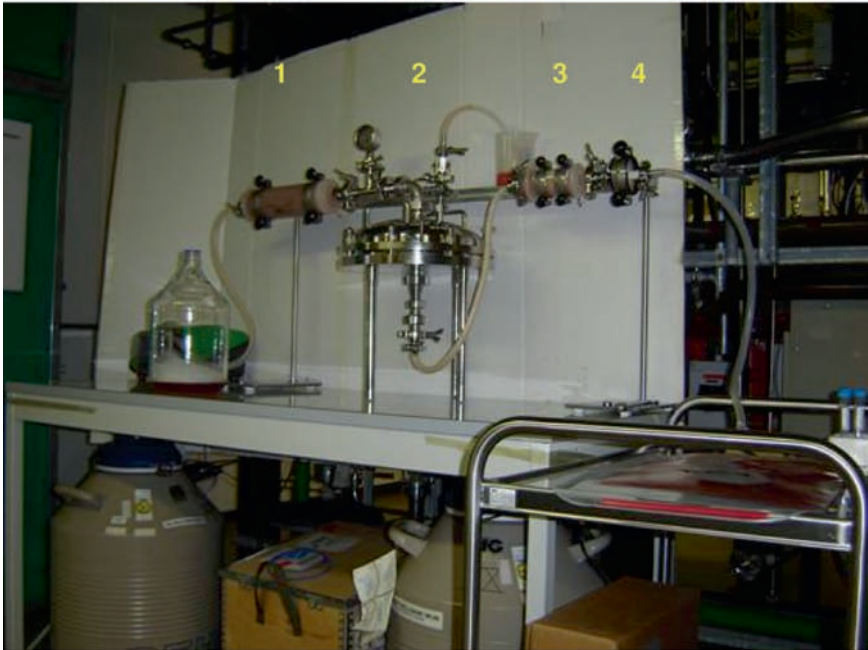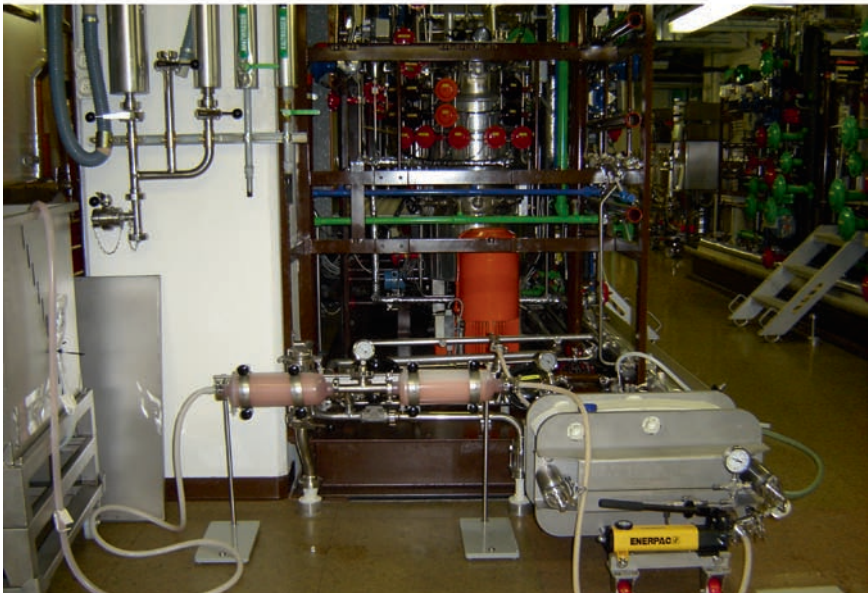
Fig. 9a,b Harvest setups used for 10-L and 100-L bioreactors. **a** For the harvest of a 10-L bioreactor, a sequence of three consecutive depth filters (1–3) is installed followed by sterile filtration (4) into a harvest bag. Except for step 2, disposable filter capsules are used. **b** The harvest of 100-L bioreactors is performed using a POD® system for the depth filtration. The performance of the POD® system is such that filtration steps 1 and 2 of the depth filtration (see **a**) could be combined. The POD® cassettes are disposed after use, for the third step of the clarification as well as for the sterile filtration, disposable filter capsules are used

personnel had to be hired. A publication evaluating the economic impact of the retrofit is actually in press [11].

Taken together, these outcomes show that disposable strategies used in combination with existing facilities can be used for easy, fast and successful implementation of cell culture processes. Furthermore, the high number of different disposable options in various functional areas transformed the pilot plant into a very flexible production unit able to cope with different project requirements in the future.

3   Case Study II: Media Preparation Merck Serono, Switzerland

3.1   *Abstract*

In order to increase the capacity of a mammalian cell process, Merck Serono compared conventional technology to disposable technology for media preparation and storage from many viewpoints. This comparison, highlighting advantages of disposable technology, enabled productivity to be increased by reducing the number of media preparations. In the case studied, the optimal configuration was a hybrid installation combining stainless steel vessels with plastic bags.

3.2   *Frame of Case Study II*

As the number of medicines in the pipeline increases, the biopharmaceutical industry is entering a phase where production demand outstrips available capacity. Despite the number of projects, there is currently a serious capacity shortfall that is likely to grow significantly by 2012. As a consequence, pharmaceutical companies have gained increased interest in disposable technologies. One of the world's leading manufacturers of biopharmaceuticals, Merck Serono, with numerous facilities worldwide is increasingly turning to new disposable technologies.

Pharmaceutical companies cover a wide range of activities such as process development, process scale up, validation and cGMP manufacturing. Due to the multi-product nature of manufacturing, the company has decided to integrate disposable technology into their production facilities. Current biopharmaceutical manufacturing techniques tend to be based on traditional fermentation and purification techniques that involve large, stainless steel, fixed facilities. These conventional operations are complex, involving multiple, time-consuming steps such as reactor assembly, cleaning, and aseptic preparation. Also, a high level of expertise is required to avoid the dangers of contamination. A substantial amount of investment in time and money, as well as an effective infrastructure is thus required to maintain the necessary conditions and installations at the plant. As a result, it is often difficult

to make process changes once the installations are fully established. With regard to this, disposable technology may offer a solution to this situation by offering a large number of advantages over conventional operations.

Due to the increasing demand for more flexible and cost-effective manufacturing capacity driven by this growing market, the concept of disposable technology has been carried through at Merck Serono, Switzerland. The company recently designed, constructed, installed and validated a disposable system so as to adapt more swiftly to a very demanding market. Comparisons between conventional and disposable facilities in terms of economic costs, cleaning-in-place (CIP) and sterilization-in-place (SIP) were specially conducted for media preparation and storage (Case study No. II) as well as for buffer preparation (Case study No. III).

3.3   Process Production Description

Figure 10 demonstrates the different steps required in a typical mammalian production process at Merck Serono.

The current case study deals specifically with the use of disposables for large-scale production processes conducted in the pharmaceutical facility. Twenty years ago, the company developed a mammalian perfusion based-process for the production of a fertility hormone. The production process is described in Fig. 11.

Until the year 2000, the production of the fertility hormone was performed in a different area than the one used today and corresponds to the initial configuration that was used for the process. This area was equipped with a 250-L bioreactor and a 50-L bioreactor. The whole upstream production process lasts 43 days, including 2 growth phases in 50-L bioreactor and 250-L bioreactor, one rinsing phase and one production phase. The media for both bioreactors was prepared in a 1,000-L stirred tank and stored in a 1,200-L stirred tank. The former process using all these installations allowed six runs maximum to be performed per year, each lasting 43 days.

In 2000, the company decided to increase the capacity of production of the fertility hormone. To improve the efficiency and economics of the production process, a study
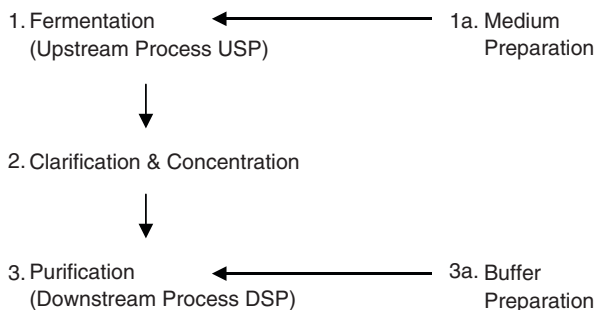
Fig. 10 Flow chart of a mammalian production process
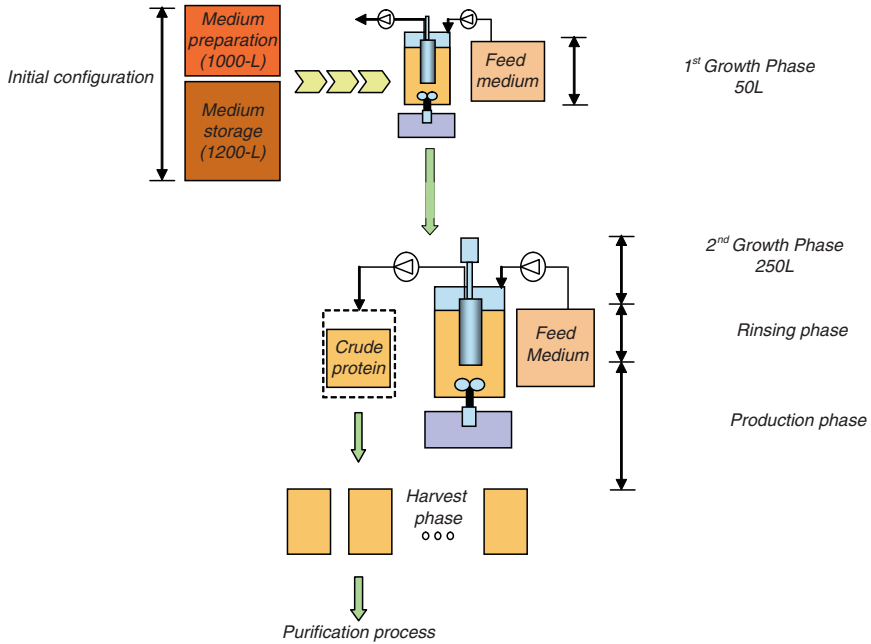
Fig. 11 Former upstream process flow diagram of protein manufacturing at the Merck Serono facility

comparing stainless steel, fixed installations to disposables has been recently conducted at the manufacturing site. The study permitted defining the best production strategy that would increase the volume of fertility hormone produced per year.

Given that the goal of the strategy was to increase the number of runs per year, that is to say increase the capacity of production of the fertility hormone; the company decided to build a new area. To support further the increase of capacity a combination of one 50-L bioreactor and two 250-L bioreactors for the production process was installed.

Moreover, concerning the media preparation and storage, two options were analyzed, so-called configuration A and configuration B:

- *Configuration A*, using stainless steel vessels: a 1,000-L stainless tank and a 2,400-L stainless tank were respectively required for media preparation and storage
- *Configuration B*, using disposables: a 2,500-L stainless tank and six 800-L disposable tanks were needed in this case for media preparation and storage, respectively

Both new configurations A and B are described in Figs. 12 and 13, respectively.

In case study No. II, media preparation is the step of the production process in which disposable technology is compared to traditional installations in terms of economic costs of operation (such as preparation and storage of support containers), cleaning in place (CIP) as well as sterilization in place (SIP).
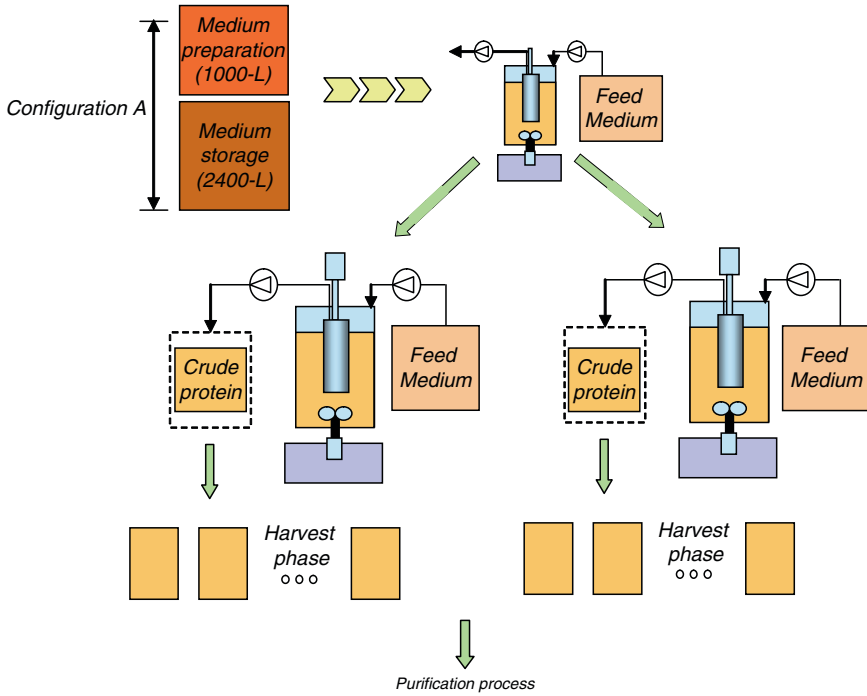
Fig. 12 New upstream process flow diagram protein manufacturing in Merck Serono facility – configuration A

3.4 Costs of Installation

3.4.1 Media Preparation

In the case of configuration A, the average costs for a 1,000-L preparation tank that is necessary to set up the new media preparation unit reaches €43,500 (prices dating from 2001). Assembly and civil engineering, including preparations of the site with foundations, as well as implementation of the equipment are estimated at 60% of the costs of the main material. Costs corresponding to assembly as well as civil engineering thus reach €26,100. Indirect costs of the construction site, including transport of equipments of construction, as well as taxes, insurances and diverse loads of construction site are also to be taken in account, reaching 80% of the costs of the preparation tank, €34,800. Therefore, the total costs for the 1,000-L preparation tank come to €104,400.

For configuration B, a similar evaluation was performed for the determination of average costs of the 2,500-L preparation tank. Average costs of equipment reach €55,300, assembly and civil engineering as well as indirect costs of the site construction are €33,200 and €44,200, respectively. Therefore, the total costs for the 2,500-L preparation tank come to €132,700.
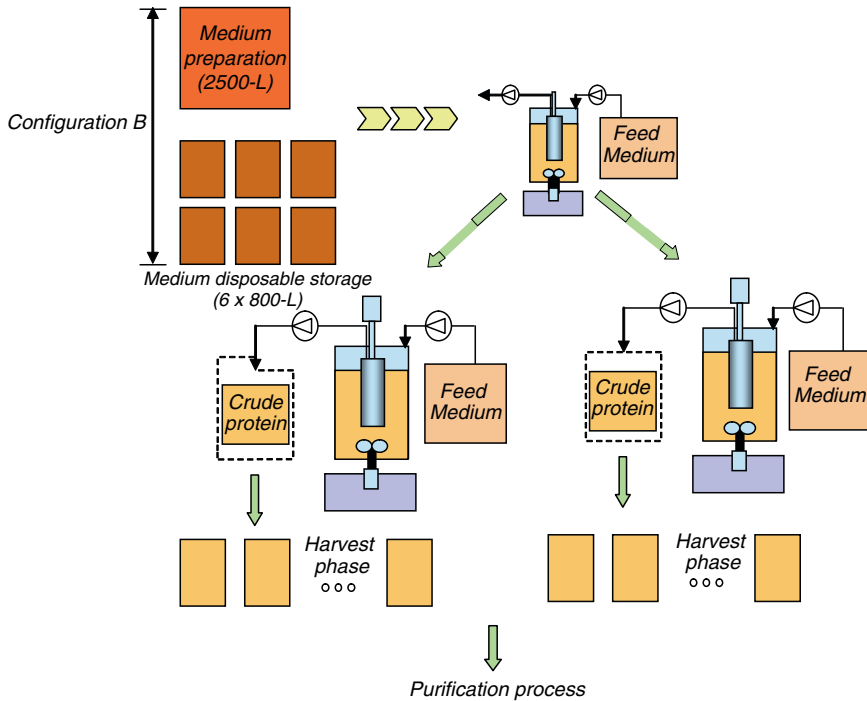
Fig. 13 New upstream process flow diagram protein manufacturing in Merck Serono facility – configuration B

3.4.2 Media Storage

Applying a similar reasoning for the storage taking in account assembly, civil engineering as well as indirect costs, the total costs of a 2,400-L storage tank in the configuration A come to €122,100.

In the same way, the total costs for the storage of the media in six disposable 800-L tanks in case of the configuration B reach €54,900.

The different costs of equipment, including assembly, civil engineering as well as indirect costs for media preparations as well as storage are reported in Table 1.

The media preparation capacity increase allowed with the configuration B for media preparation appears to be only 27% more expensive than configuration A. Moreover, even if the prices of the bags required for six storage tanks in configuration B are not taken in account, this configuration allows doubling of media storage volumes in comparison to configuration A and appears to be 2.2-fold cheaper.

As a result, configuration B using disposables, including preparation and storage tanks, is 21% less expensive than the total costs needed for the installation of configuration A using fixed, stainless steel, hard-piped facilities.

Table 1 Average costs for media preparation and storage for the set up of new production unit at the Merck Serono facility

Media preparation	Configuration A (1,000-L tank)	Configuration B (2,500-L tank)
Costs of raw material (€)	43,500	55,300
Civil engineering (€)	26,100	33,200
Indirect costs (€)	34,800	44,200
Total preparation costs (€)	104,400	132,700

Media storage	Configuration A (2,400-L tank)	Configuration B (six 800-L tanks)
Costs of raw material (€)	50,900	22,900
Civil engineering (€)	30,500	13,700
Indirect costs (€)	40,700	18,300
Total storage costs (€)	122,100	54,900
Total costs (€) (preparation and storage)	226,500	187,600

3.5 Production Capacity of Installations A and B

Increase of manufacturing capacity of the fertility hormone was also reached by tightening the manufacturing schedule. By using 2 250-L bioreactors a maximum of 16 runs, each lasting 43 days plus 6 days of preparation, could be performed per year.

The two configurations have been assessed to verify their potential to support the increase of the number of manufacturing runs per year. The assessment showed that the numbers of media preparation needed per year are not same for configuration A and B as the medium storage capacity is significantly smaller in configuration A. This difference has a direct impact on the annual CIP and SIP costs.

To compare further both tested installations, the annual CIP and SIP costs for configurations A and B are evaluated in the following part of the study. After each medium preparation, the vessels have to be cleaned with sodium hydroxide and rinsed with purified water and Water For Injection (WFI) in both configurations. The medium preparation tanks are sanitized periodically once a month at 124 °C for 30 min. Therefore, the impact on the operation costs of medium preparation tanks is linked to the number of medium preparation and CIP cycles performed.

3.5.1 Configuration A

The important number of runs per year (16) requires that the storage tank has to be used continuously. Therefore, the tank can be neither cleaned nor sterilized in place. It is therefore obvious that the planning, as previously described, performing 16 run per year, is completely theoretical for configuration A. Such a production schedule cannot be implemented for routine manufacturing due to the resulting high contamination risks of the storage tank that could result in the stopping of the both production bioreactors.

Cleaning in Place

To conduct 16 runs per year, the use of configuration A requires 224 1,000-L media preparations. Indeed, a media preparation is conducted each 3 running days and one production run lasts 43 days. As a result, 14 preparations are thus performed per run, and 224 preparations are then needed per year (16 runs).

One CIP being performed after media preparation, configuration A needs 14 CIP per run, therefore 224 CIP per year. CIP thus involves the costs as given in Table 2.

According to Table 2, the annual CIP costs of the 1,000-L preparation tank used for configuration A amount to €49,800.

Sterilization in Place

In both tested configurations A and B, the SIP installation is capable of withstanding steam pressures up to 20 psi and corresponding sterilizing temperatures in the 121–125 °C range for 30 min. Once sterilization is performed, water at 15 °C is then injected into the double envelope to cool the tank down.

During the sterilization step, the quantity of heat provided by the steam must be sufficient to raise the temperature inside the tank, the accessories, and piping from 15 to 124 °C as well as to maintain this temperature right through the sterilization process. The following calculation has been applied to determine the necessary quantity of steam ($m_{steam\,tot}$) to provide for SIP:

$$m_{steam\,tot} = m_{steam\,1} + m_{steam\,2} + m_{steam\,3}$$

with:

$m_{steam\,1}$: steam quantity to be injected into the reactor depending of stainless steel of the bioreactor, accessories and piping;

$m_{steam\,2}$: steam quantity to compensate thermal loss during sterilization at 124 °C;

$m_{steam\,3}$: steam quantity to get rid off the air from the reactor.

The quantities of steam required for the SIP of the 1,000-L preparation tank have been reported in Table 3. Given that the utilization of the tank requires one SIP per month, annual costs of SIP have also been indicated in the table.

Table 2 Determination of annual CIP costs for the 1,000-L tank in configuration A

Configuration A	Quantity (L)			Costs (€)	
Reagents	Per preparation	Per run (14 preps)	Per year (16 runs)	Units costs (€)	Total costs (€)
WFI	250	3,500	56,000	0.5 € L^{-1}	28,000
Na$_2$CO$_3$ 0.5 N	250	3,500	56,000	0.38 € L^{-1}	21,300
Purified water	750	10,500	168,000	3.01 € m^{-3}	500
Total costs (€)					49,800

Table 3 Determination of annual SIP costs for the 1,000-L tank in configuration A

Configuration A		Quantity (kg)	Costs (€)[a]
Steam	Per month	Per year (12 sterilization)	Annual costs (€)
$m_{steam\ 1}$	12	144	14,000
$m_{steam\ 2}$	2	24	2,300
$m_{steam\ 3}$	1.5	18	1,700
$m_{steam\ tot}$	15.5	186	18,000
Total costs (€)			18,000

[a]Costs of steam: €97 ton^{-1}

According to the table, annual SIP costs of the 1,000-L preparation tank needed for configuration using fixed facilities reach €18,000.

Total annual costs total €67,800, with CIP and SIP costs reaching €49,800 and €18,000, respectively.

3.5.2 Configuration B

A 2,500-L container was used for media preparation and 775-L Hyclone® bags were specially adapted to the six 800-L-support containers for intermediate storages. Bags are maintained sterile all production long; no CIP and no SIP are then required for disposable facilities. CIP costs in this configuration are thus reduced to the costs relative to the stirred 2,500-L preparation tank.

Cleaning in Place

One media preparation being conducted each 3 running days and one production run lasting 43 days; 6 media preparations per run are thus needed in configuration B. The use of this hybrid installation then requires 96 CIP per year. Annual CIP costs are reported in Table 4.

According to Table 4, CI P costs of the 2,500-L preparation tank reach €21,300.

Table 4 Determination of annual CIP costs of 2,500-L tank in configuration B

Configuration B		Quantity (L)		Costs (€)	
Product	Per preparation	Per run (6 preps)	Per year (16 runs)	Units costs (€)	Total costs (€)
WFI	250	1,500	24,000	€0.5 L^{-1}	12,000
Na_2CO_3 0.5 N	250	1,500	24,000	€0.38 L^{-1}	9,100
Purified water	750	4,500	72,000	€3.01 m^{-3}	200
Total costs (€)					21,300

Sterilization in Place

A similar evaluation for determining annual SIP costs has been applied with the determination of $m_{steam\ 1}$, $m_{steam\ 2}$, $m_{steam\ 3}$ and $m_{steam\ tot}$.

Quantities of steam required for the SIP of the 2,500-L preparation tank have been reported in Table 5. Knowing the cost of steam (€97 per ton), annual SIP costs have also been described in Table Table Table 5.

In configuration B using disposables for the six 800-L storage tanks, SIP costs are reduced to the sterilization of the 2,500-L preparation tank. SIP costs thus reaching €47,000 are 2.6 times more important than SIP costs as described for configuration A (€18,000).

Despite the higher SIP costs in the hybrid configuration, the study demonstrates that the configuration using disposable equipment significantly minimizes the cleaning-in-place. Actually, total annual CIP costs in this new configuration are reduced *2.3 times* compared to configuration A using fixed, stainless steel, hard-piped facilities.

3.6 Conclusions of Case Study II

Over the past decade, disposable technologies have been increasingly used to improve the efficiency and economics of process development and production. There are actually a number of key applications where disposable technology appears particularly beneficial.

This case study being conducted at Merck Serono shows that disposable technology plays an important role, and this in each processing step of a pharmaceutical product (here only media preparation and storage have been described in case study No. II). Instead of fixed, stainless steel, hard-piped facilities for manufacturing biopharmaceuticals, single-use, disposable technology is used to create portable, flexible manufacturing systems. Then the use of disposable components and production-scale disposable stirred tank bioreactor systems appears to provide flexibility to produce several different products at a single facility. The new technology also allows manufacturing capacity to be established rapidly at reduced cost compared to traditional systems.

Table 5 Calculation of quantities of steam required for SIP of 2,500-L tank in configuration B and determination of annual SIP costs

Configuration B		Quantity (kg)	Costs (€)[a]
Steam	Per month	Per year (12 sterilization)	Annual costs (€)
$m_{steam\ 1}$	32	384	37,200
$m_{steam\ 2}$	5	60	5,800
$m_{steam\ 3}$	4	48	4,700
$m_{steam\ tot}$	41	492	47,700
Total costs (€)			47,700

[a] Costs of steam: €97 ton^{-1}

Comparisons of total costs, including installation of the system, annual CIP costs as well as annual SIP costs, are reported in Table 6.

Capital and maintenance costs for disposable installation are reduced compared to stainless steel vessels and hard-piped facilities. Disposable technology also allows a significant benefit in the increase of production capacity through minimizing cleaning and revalidation time in equipment changeover. Since all product-contact surfaces are disposable, and the need for CIP and aseptic assembly of parts is eliminated. In this case study, due to the use of a larger preparation tank in the configuration using disposable technology, there is no gain in sterilization-in-place. Finally, total costs including installation of the system, cleaning and sterilization for the configuration using disposables are strongly reduced compared to the total costs required for the conventional installation.

Disposable bags can offer further advantages, including a reduction in the microbial contamination risk due to the absence of valves and manifolds traditionally used for transport of media and buffers. Furthermore, disposables give the flexibility to move rapidly from one production campaign to another, replying to specific customer requirements with no cross-contamination risk. In contrast, the contamination risk in the configuration using fixed installations would be high due to the absence of cleaning as well as sterilization of the tank in order to cope with the tightened manufacturing schedule of 16 runs per year. This absence of cleaning as well as sterilization makes configuration using fixed installations unrealistic for a real industrial process where safety appears as a central point of concern. Therefore, a traditional installation would not allow an increase of production capacity at the same extent as the disposable installation.

Disposable configuration also allows a three times reduction in work force. Indeed, configuration using fixed facilities requires 224 media preparations per year (one preparation per day). In contrast, in the disposable configuration, the protein process with 16 runs per year only requires 96 media preparations per year (one preparation per 3 days).

Finally, disposable technology has advantages that are not only beneficial for multi-product facilities, as demonstrated at Merck Serono. Reduced capital investment, reduced cleaning and validation time, reduced contamination risk, reduced work force and optimization of production capacity are equally advantageous for single-product plants.

Disposable technology not only increases productivity but also reduces the time required for initial facility installation and validation requirements. As a result, companies can bring their drugs to the market more swiftly.

Table 6 Comparisons of total costs, including installation, CIP and SIP between configuration A and B

	Configuration A	Configuration B	Savings
Installation costs (€)	226,500	187,600	1.21
CIP costs (€)	49,800	21,300	2.34
SIP costs (€)	18,000	47,700	−2.65
Total costs (€)	294,300	256,600	1.15

4   Case Study III: Buffer Preparation at Merck Serono, Switzerland

4.1   Abstract

Merck Serono used a similar approach for the installation of a buffer preparation and storage unit as described before for the media preparation and storage unit. Until now, the installation was composed of fixed, stainless steel, hard-piped facilities. The experience acquired form the media preparation and storage study lead the company to implement an equivalent hybrid installation for buffer preparation and storage combining stainless-steel vessels and disposables.

4.2   Frame of Case Study III

Previous installations consisted of one 800-L preparation stirred tank and six 800-L storage stirred tanks. After each buffer preparation, the preparation tank was rinsed with purified water and WFI and periodically sanitized. Likewise, storage vessels were sanitized and cleaned when buffers were changed.

   As used for media preparation and storage units, a new hybrid configuration was installed within the buffer unit. This new configuration was composed of a fixed 1,000-L preparation tank and nine 800-L disposable storage tanks. A study similar to the media preparation installation was also conducted for the buffer preparation and storage unit. The study underlined identical advantages brought by the new hybrid configuration, such as reduction of capital investment, reduction of cleaning and validation time, reduction of contamination risk as well as reduction of work force. In contrast to the results of case study II, an extra advantage became obvious in the fact that storage tanks with the integrated disposable bags could be installed in stacks. This reduced the footprint in comparison with a traditional installation resulted in a gain of space of almost 50% with the new hybrid configuration.

5   Case Study IV: Comparison of Disposable vs Traditional Technology for a 2,000-L Pilot Plant

5.1   Abstract

The development of stirred disposable bioreactors that can be operated with significant volumes prompted a direct comparison of the new disposable with the conventional technology. This comparative study was carried out on paper and a monoclonal antibody manufacturing process was taken as the basis for the design of a fully

disposable and a completely conventional pilot plant. The aim of this study was to determine the potential advantages (technical, economical and operational) that the new technology could offer with regard to the conventional one.

5.2   Introduction

In early 2007, a project comparing the conventional technology (stainless steel) and the new disposable technology (plastic) was carried out in the context of a pilot plant for the process transfer of a monoclonal antibody. This pilot plant was evaluated with two types of equipment, conventional equipment consisting of stainless steel bioreactors and disposable equipment consisting of plastic bioreactors and materials. The implementation of these two types of pilot plants allowed the comparison of these two technologies and the highlighting of the interest of the emerging disposable technology for such a project.

The aim of this project was to compare technically and economically the two technologies put into application in a pilot plant in order to highlight the potential of the emerging one.

The model process consists of three phases using six different bioreactor volumes. The production step is performed in fed-batch mode. The three phases are:

- A *seed train* allowing the creation of a cellular stock in order to have the production cycles renewed on a regular basis
- An *inoculum train* with the purpose of increasing the biomass before production

A *production phase* with the purpose of obtaining a large amount of monoclonal antibodies (Fig. 14).

At the end of the fermentation process, the culture fluid is harvested in order to separate the cells and cellular debris from the culture containing the monoclonal antibodies. This step is operated in a different way according to the type of pilot plant. For the conventional pilot plant, a continuous disc centrifuge performs the harvest. For the emergent technology, a disposable filtration device is used.

5.3   Choice of Equipment

5.3.1   Bioreactors

In order to be able to compare the two technologies in an optimal way, all parameters and settings used (mainly the size of the bioreactors, the scale up factors, and the culture mode) were maintained, whenever possible, between the two types of installations. The comparison was performed from the thawing of a Working Cell Bank (WCB) ampoule to the harvest of the production run. This workflow therefore allowed highlighting of the essential differences between the two types of installations.

Fig. 14 Flow chart of the model process covering all steps from the WCB thawing to the harvest of the production bioreactor. Three phases can be distinguished, These are the seed train, the inoculum train and the production train, respectively
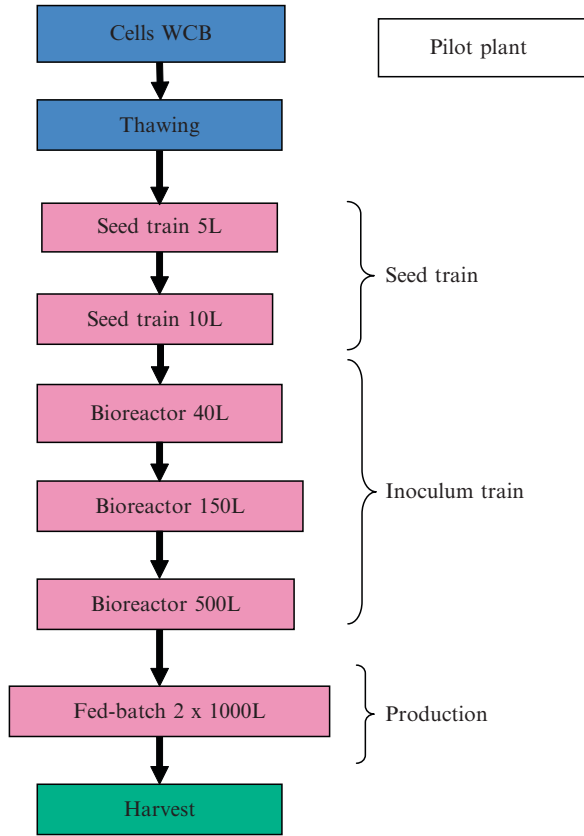
The disposable bioreactors chosen for this project were those commercialized by Xcellerex, USA. At the time, very few suppliers proposed fully equipped disposable bioreactors: mainly Hyclone and Xcellerex. The reason we have chosen the second was based on the fact that the production step was supposed to be performed in a 2,000-L bioreactor. The volumes of the Xcellerex equipment already available were more adapted to our process (up to 1,000-L) as the Hyclone setup (up to 250-L, the 1,000-L was not expected until April 2007). In fact, at the time, the volumes commercialized by Xcellerex were 200-L, 500-L and 1,000-L. For the seed train, a 10-L bioreactor was necessary, and a 50-L bioreactor for the first step of the inoculum train. Xcellerex agreed to look at the possibility of custom making a 10-L and a 50-L bioreactor especially for us. Therefore, the following bioreactors were used in this study: a 10-L bioreactor for the seed train: a 50-L, a 200-L and a 500-L for the inoculum train and two 1,000-L bioreactors for the production step. Being limited by the disposable bioreactors sizes available on the market, the stainless steel bioreactors used were chosen from the company Sartorius, the volumes of the bioreactors being similar to the disposable ones. A comparison of the chosen bioreactors is shown in Table 7.

Table 7 Bioreactors chosen for the pilot plant process for the disposable and conventional technologies

Step	Working volume (L)	Stainless steel		Disposable	
		Bioreactor	Total volume (L)	Bioreactor	Total volume (L)
Seed train	5	BIOSTAT D-10	13	XDR-10	13
	10	BIOSTAT D-10	13	XDR-10	13
Inoculum train	40	BIOSTAT D-50	66	XDR-50	65
	150	200 L	264	XDR-200	260
	500	500 L	660	XDR-500	650
Production	2,000 (2 × 1,000)	1,000 L	1,320	XDR-1000	1,300

5.3.2 Harvest

For the conventional pilot plant, several methods were available for the harvest of the final fermentation. A continuous disc centrifuge was chosen, as it shows a lot of advantages with regard to our process. Indeed, the biomass is separated progressively during the centrifugation; there is no further need to stop the equipment to clean it. The centrifuge chosen was the disc centrifuge CSC6 (Westfalia); it treats 100-L of liquid per hour. In order to process the 2,000-L of culture media, this step takes 20 h.

For the disposable facility, the filtration device commercialized by Millipore was chosen. The POD® are highly performing filters conceived for the primary or secondary clarification steps of pilot and industrial scale processes. This system can be completely modulated, thus giving a huge flexibility to the user. In fact, the modularity of the system allows several filtration cassettes (up to 10) to be combined in order to treat any possible volume. The system can support from one 0.11- to ten 1.1-m^2 POD® cassettes. In order to treat our harvested volume, ten POD® filter cassettes of 1.1 m^2 will be necessary (Table 8). The primary clarification is followed by a 0.2-mm filtration.

As is shown in Table 8, processing our 2,000 L of harvest is 24 times quicker (50 min compared with 20 h) with the disposable device than with the conventional one. Furthermore, the use of a disposable filtration device exempts and/or reduces an eventual SIP and CIP cycle, thus making the use of this technology even more interesting.

5.4 Comparison

5.4.1 Cleaning in Place/Sterilization in Place

A certain advantage of the disposable technology with regard to the conventional one is the lack of any cleaning in place (CIP) sequence of the material employed for the process. This non-deniable gain of time comes together with an economical

Table 8 Harvest system chosen for the pilot plant process for the disposable and conventional technologies and process time comparison

	Conventional	Disposable
Equipment	Disc centrifuge CSC6 (Westfalia)	10 POD of 1.1 m² (millipore)
Time to treat the 2,000 L of culture media	20 h	20 min + 30 min of 0.2-mL filtration

interest as for the water and energy consumption. Furthermore, there is no sterilization step to consider, and thus a considerable reduction of the use of steam, water and therefore energy. Also, the cross contamination risk is considerably reduced, as all the material is single use. No cleaning validation is therefore necessary, thus avoiding the time and resources spent on this activity. All activities were facilitated by the integration of a simple "plug-and-play" approach.

The time granted to the implementation of the disposable facility is considerably inferior to that required for the implementation of the conventional facility, the latest needing the complex arrangement of water and steam piping (pipes, boiler, collection and treatment of the used water). Due to the absence of such installations, the set-up of a single use facility needs considerably less space than a conventional one.

5.4.2 Spent Time Comparison

If the time spent for each step of the pilot plant is compared for the two technologies (Fig. 15), it appears that the use of single-use technologies leads to a time reduction when compared to conventional technology. Indeed, every step (from the seed train to production) is performed in much less time (~5% of time reduction on average). If the detail of each step is analyzed, it appears that this difference is mainly due to the lack of SIP and CIP. Concerning the harvest, the time reduction is much more considerable (~61%). This is due to the use of completely different harvesting technologies: one is a centrifuge, where even if the culture is treated continuously, the time necessary to process a certain quantity of liquid remains considerable (20 h for 2,000 L of culture media); the other one is a filtering device called POD®, composed of several modules that can be added according to the quantity of liquid to process. This technique is therefore much more efficient and fast (50 min). This difference is not that evident in Table 8 as the harvest step takes into consideration the draining of the bioreactors as well as the cleaning if applicable.

A Gantt chart for each technology was elaborated. When the two Gantt charts were compared, they seemed to have the same profile. However, the duration of some main activities appear to be very different between the two technologies. In fact, some of these steps are considerably reduced in the chart for the disposable technology with regard to the conventional one. For example, when installing or removing a bag instead of performing an SIP or CIP, the time gain is 3-fold and 12-fold respectively. Furthermore, when installing the pilot plant for the first time,

Stainless Steel

| Time (hours) | 0,2 | 710,18 | 271,08 | 691,7 | 39,6 |

Plastic

| Time (hours) | 0,2 | 699,1 | 244,1 | 678,2 | 15,3 |
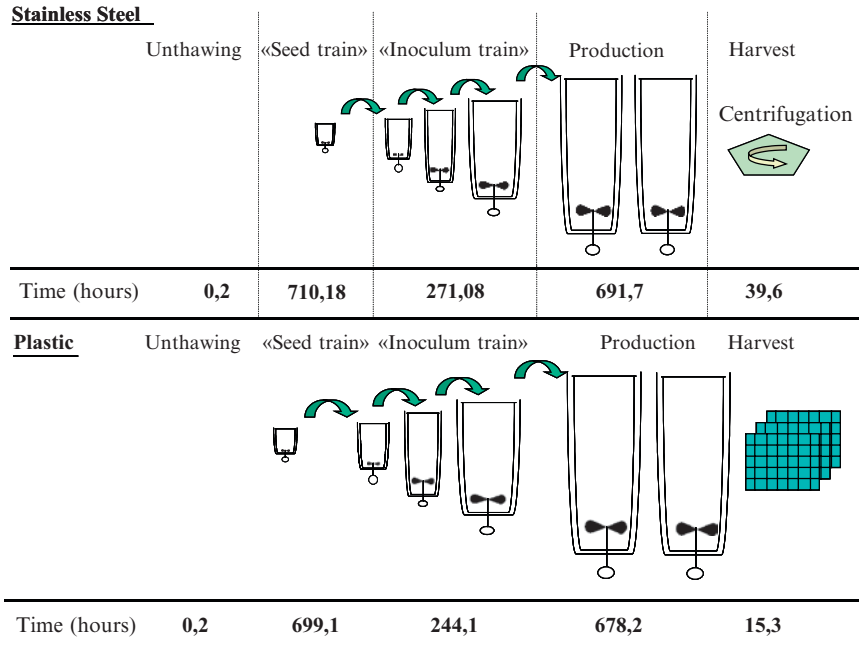
Fig. 15 Schematic representation of the process for the conventional (stainless steel) and emerging (disposable) technology with the times taken by each step

the time gain for the disposable technology was estimated to be seven times less than for conventional technology.

5.5 Economic Evaluation

Explanation of the different amounts calculated:

- The *Investment charges in Units* are the investments in production units, the implementation investments and storage
- The *Fixed Capital* is the engineering costs, the replacement parts stock storage, and the costs due to the use of a patented technology during the process
- The *Depreciable Capital* is the fixed capital plus the initial costs, the lending fees, and the launching fees
- The *Total Investment* represents the Depreciable Capital plus the working capital fund. The working capital fund depends mainly on the variable charges
- The *Fixed Charges* include the depreciation, the financial costs, the up keeping and maintenance costs, the taxes and insurances, the general and administrative costs and the renting costs

- The *Variable Charges* include the media, the reagents and the utilities
- The *Operating Costs* include the fixed charges, the variable charges and the work force

The complete economical evaluation of the two types of installations allowed us to highlight some significant differences concerning some economic ratios.

First, an important thing to notice is the fact that the cost of the main equipment for the implementation of the disposable pilot plant is lower than the one of the conventional pilot plant (€1,743,533 against €2,541,440). Furthermore, the total investment is also lower for the disposable technology (see Table 9: €8,184,403 against €12,057,171).

Moreover, the fixed charges and even the workforce charges tend towards the same direction. Indeed, the savings regarding the utilities costs (water, steam, electricity) aren't insignificant (€26,451 against €48,981). This difference correlated with our expectations, due to the absence of any cleaning process of the equipment used. Regarding the variable charges, the disposable technology is more expensive than the conventional one (−85%). However, this big difference doesn't make the disposable technology less interesting as even with this big difference, the operating costs are still much more profitable when using the disposable technology.

The real minimum selling price of the model process antibody established for the disposable technology (€289,591 kg^{-1}) is almost half that obtained when using the conventional equipment (€412,787 kg^{-1}). This constitutes a real economic advantage that is further supported by the fact that the disposable technology Pilot Plant crossed the limit of profitability (15.1% against 13.8%) already at the end of the project timeline of 12 months. This means that investment at such a scale

Table 9 Comparison of the different expenses when using the conventional or disposable technology

		Conventional technology	Disposable technology	% Savings
Investment and operational expenses	Investment charges in units (IU)	9,052,610	5,927,141	35
	Fixed capital (FC = $I_3 + I_4 + I_5$)	10,953,658	7,171,840	35
	Depreciable capital (DC = FC + $I_6 + I_7 + I_8$)	11,832,705	7,844,047	34
	Working capital fund ($I_9 = 2 \times I_8$)	224,466	340,356	−34
	Total investment (TI = DC + I_9)	12,057,170	8,184,403	32
Operational cost and operating expenses	Fixed charges per year	12,466,132	8,258,947	34
	Variable charges	104,582	697,409	−85
	Workforce charges	568,815	323,669	43
	Operating costs	13,139,529	9,280,025	23
Project profitability	Minimum selling price (€ kg^{-1})	412.787	289.591	30
	Internal profitability rate (%)	13.8	15.1	–

induces a lower initial financial contribution when using the disposable technology, which could be a decision-making argument when discussing the project.

The re-evaluation of the internal profitability rate (23% against 19.6%) confirms the fact that this disposable option has a non-deniable economic advantage.

However, it is important to notice that all of our calculations were done at a pilot scale (project timeline: 12 months). Even in such short period, the advantages of the disposable technology could be highlighted and were significant. We can imagine that at a larger production scale the advantage could only be improved. It could therefore be interesting to compare these two types of technologies over several years, in order to determine the advantage of the disposable technology at long period.

6   Conclusion Disposable Technology: Pros and Cons

In the case studies, the various possibilities of disposable strategy application in biotechnology are outlined. In case study I, a retrofit of an existing microbial fermentation facility for mammalian fermentation in a very short timeline was presented. All operations of the upstream process were performed following a disposable strategy and the bioreactor was the only traditional equipment left. The disposable strategy allowed in this case a significant reduction in the lead-time of the project. No shut down for refurbishment and no costly adaptation of utilities of the pilot plant were needed. The workload for qualification and validation was significantly reduced. The choice of mobile systems and custom-made manifolds used for media preparation, bioreactor operation and harvest gives furthermore maximum flexibility for the future use of this pilot plant as manifolds can easily be customized. Product changeover can be performed rapidly due to the absence of lengthy cleaning validations. The major disadvantages of following this strategy were the dependence of suppliers, the time needed to design the different manifolds and the increased effort for warehousing. Questions regarding extractables and leachables from disposable materials have to be evaluated carefully. It is expected that these questions will be resolved definitively in the near future.

The need to increase production capacity by reducing costs installation triggered a large interest for the companies in disposable technology. The case studies II and III describe two novel flexible plastic-based disposable facilities that have been recently implemented in a Switzerland pharmaceutical company. In both facilities studied, the media and buffer preparation and storage are based on an interesting hybrid system combining disposable as well as stainless-steel vessels. The advantages of these disposable hybrid systems compared to the conventional technology, carrying stainless fixed vessels only, include lower cost, ease of use, flexibility, and efficient aseptic transfers via thermoplastic tube welding. Moreover, one part of the vessels being disposable and pre-sterilized before use, cleaning, sterilization, and maintenance operations are strongly reduced or eliminated. More importantly, this reduction of cleaning-in-place can significantly increase productivity, enhance sterility insurance, improve operator safety, and speed time-to-market. Disposable technology appears

therefore to be efficient and low cost media and buffer systems and is applicable to various cell cultures at small- to large-scale. Due to its simplicity (no cleaning or maintenance operations), disposable technology leads to the development of innovative and groundbreaking equipment that can be used in the different steps of production processes, such as cellular amplification using bags technology. With regard to this, for instance, numerous disposable biosensors can now be used for a suitable cell culture monitoring, offering several large benefits due to their simplicity and rapid response as a promising device for the advances of disposable technology in large-scale processes.

In case study IV, a comparison between the disposable and conventional technology was made in the frame of a mammalian fermentation pilot plant scale-up for the process transfer of a monoclonal antibody. The two technologies were therefore compared from many angles such as the technical, time gaining advantages, the economical advantages, the number of technicians needed, etc. It therefore appeared that a clear advantage of the use of disposable technology was the lack of CIP and SIP steps during the process. This advantage reflects a gain in the matter of time and also in the matter of the quantity of utilities used. Furthermore, the time needed for the implementation of the pilot plant is considerably reduced when using disposable material, making the disposable technology interesting when companies want to implement a process in a very short time frame. In fact, the disposable pilot plant gave an internal profitability rate of 23% instead of 19.6% for the conventional one on a 1 year basis; thus, besides being paid off after 1 year, the pay off is better than for a conventional technology pilot plant. Finally, in correlation with the economical advantages, the disposable technology pilot plant needs a smaller working force for all indirect activities such as quality control for cleaning and SIP validation.

Taken together, the four case studies outline various applications for disposable technology being used either as replacement of or in combination with traditional technology. Using disposables had in all case studies a positive impact on project success and overall project costs. These results are based on the intrinsic versatility of disposable devices providing a high potential for customization and the reduction of installation and operating costs.

These advantages favor the wide use of disposable technology in an environment of increasing pressure for competitive R&D and manufacturing costs.

References

1. Pavlou AK, Reichert JM (2004) Nat Biotechnol 12:1513
2. Zika E, Papatryfon I et al (2007) Consequences, opportunities and challenges of modern biotechnology for Europe. Report EUR 22728 EN, http://www.jrc.ec.europa.eu/Accessed 03 January 2008

3. Lawrence S (2005) Nat Biotechnol 23:1466
4. Pavlou AK, Belsey M (2005) Eur J Pharm Biopharm 59:389
5. Walsh G (2006) Nat Biotechnol 7:769
6. Butler M (2005) Appl Microbiol Biotechnol 68:283
7. Hodge G (2004) Biopharm Int 3:38
8. Cardona M, Allen B (2006) Bioprocess Int 4:10
9. Wrankmore M (2005) Application of disposable technologies in biopharmaceutical manufacturing. BMD Summit, Disposables for Biopharm Production, Reston, VA
10. Koch A, Wittische C et al. (2006) Am Pharm Rev 7:44
11. Foulon A, Trach F et al. (2008) Bioprocess Int 6:6

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