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From the Editor

This special issue has come about through a long collaborative process with our contacts at Sartorius Stedim Biotech in Göttingen, Germany. It is the third such sponsored issue that BPI has published over the years with the company and the first one that we have produced in-house. We could not be happier with the results.

The aim here is to highlight technologies and responses to key industry challenges rather than focus over much on Sartorius itself. But the company's long-standing culture of developing its own and integrating acquired technologies and processes — toward its goal of providing total biomanufacturing solutions — is worth underscoring. In the two interviews in this issue you will read about a company that has a long history of investing in and positioning its technologies and related equipment for future needs, even when the current industry has not been quite ready for them yet. In this risk-averse era, such long-term thinking can require no small amount of corporate courage. Through its own R&D activities and combinations of licensing, partnering, and acquisitions the company has crafted its current identity and continues on a well-defined path.

As part of this issue's preparation, BPI publisher Brian Caine and I traveled to the Sartorius Stedim Biotech facility in Göttingen, Germany. There we interviewed Christel Fenge (vice president of marketing for fermentation technologies), Reinhard Vogt (executive vice president of marketing, sales, and services and a member of the administrative board), and Stefan Schlack (senior vice president of marketing and product management). Brian and I were grateful for the time they devoted to speaking with us — all during a very busy few days. That week the company was also conducting a two-day conference at its Sartorius College. Academic and industry speakers presented analyses of PAT tools and how those can enhance process modeling and development under the quality by design (QbD) initiative. That event highlighted Sartorius Stedim Biotech's commitment to educating and training the coming generations of bioprocessors.

Our general theme here is the development and continual optimization of upstream technologies, so articles related to fermentation issues and to development and design of single-use bioreactors make up the first half of this supplement. This continues BPI's exploration of the present and future viability of single-use technologies (highlighted recently in our April supplement). The ultimate goal for many industry insiders is to put together a completely single-use process from production through formulation, fill, and finish. Certain assurances need to be brought to the industry at large regarding one-time use of production and processing equipment. For one thing, what are these materials, really? This is a topic of increasing interest among our readers: how plastic materials and components are made and how companies are ensuring supply-chain consistency and robustness.

Herein you'll find Sartorius Stedim Biotech's solution to that particular concern — forming the second half of this issue. The company is providing and qualifying its own film by a strong cooperation with its partner, Südpack, and is taking control of associated risks in material suitability, reliability, and availability (sourcing of raw materials). Some articles in this issue explain the intricacies of that process.

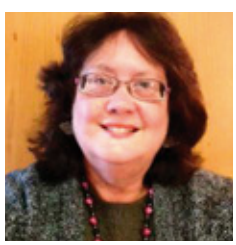
Among products highlighted in these articles and interviews is the company's new bag tester. Proper handling of single-use materials is another common theme these days. Users want to prevent leaks and tears as well as ensure bag compatibility with the products that come into contact with them. The bag tester is yet another element of the company's goal to provide total solutions to its customers. Along with a focus on training, offering clients such a tool should increase confidence in adoption of single-use systems overall.

The topic of scalability is often the proverbial elephant lounging in the back of the room at single-use conferences. How large can single-use bags go? The consensus in this issue is that 2,000 L may be perfectly adequate, now that titers are higher and processes can be scaled to run in parallel. This is not the last word on the topic, of course, but a number of authors herein discuss reasons for that conclusion.

One of those reasons is that with Sartorius Stedim Biotech's recent acquisition TAP Biosystems and its scalable line of ambr bioreactors, the company just doesn't see the need for larger systems. Customers can use TAP systems to effectively optimize their processes and titers. Also, many larger companies still have stainless steel equipment to which they transfer processes for large-scale manufacturing — so questions remain in the industry about what biomanufacturing companies want and truly need at later processing stages.

Thus, authors in this issue are primarily examining bioreactor design innovations, qualification and GMP development, use of those bioreactors with the company's line of Flexsafe bags, testing and qualification of the new film, and the status of sensor development. The latter will be an essential component for any totally single-use manufacturing operation in the future, when automation will be key. And although this issue focuses on upstream development, we conclude with an assessment of a single-use, filtration technology based on a diatomaceous-earth cell removal approach — just to take a quick look at the interface between upstream and downstream.

We thank the authors for contributing to this issue and for working closely with us to prepare their manuscripts for publication. We especially thank Sartorius Stedim Biotech's corporate communications manager, Dominic Grone, who coordinated this project with us, circulated review copies to the authors, arranged for and helped us conduct the two interviews, and generally helped us keep track of the many pieces and multiple sets of expectations. This is truly a Sartorius Stedim Biotech product, and we enjoyed working with this company's team to provide you with this publication.



S. Anne Montgomery

S. Anne Montgomery
editor in chief

Automated Mini Bioreactor Technology for Microbial and Mammalian Cell Culture

Flexible Strategy to Optimize Early Process Development of Biologics and Vaccines

by Mwai Ngibuini

The use of mammalian and microbial cells in the production of biologics and vaccines is well established, and the majority of the top 10 drugs are now manufactured in this way. There is a significant and growing pipeline of new biologics (**1**), which in combination with increased pressure on cost reduction and generic competition from biosimilars (**2**), means that many biopharmaceutical companies are looking for ways to improve productivity in their development laboratories to ensure that upstream processes are efficient and robust.

One of the main challenges for those companies is to minimise the high cost of goods that is typically associated with cell culture production processes to ensure the commercial viability of a therapy. For example, monoclonal antibody (MAb) therapies are required in large doses (6–12 g) to achieve clinical efficacy. That means they can cost tens of thousands of dollars per patient per year. Currently, the colorectal cancer treatments bevacizumab and cetuximab cost $20,000–30,000 for an eight-week course (**2**), well over 60 times more than comparable small-molecule therapies.

Photo 1: ambr250 scale-down bioreactor system for parallel fermentation and cell culture

Manufacturing prophylactic vaccines — which are a relatively small class in comparison with MAbs in terms of global sales value — is also challenging. Manufacturers need to produce affordable prophylactic vaccines for emerging markets, as well as for preventing seasonal influenza and responding to pandemic threats. Such cost and timeline issues are now

Figure 1: Optical density (OD) profiles of recombinant *E. coli* fermentation in the ambr250 bioreactor system, 15-L and 150-L fermentors

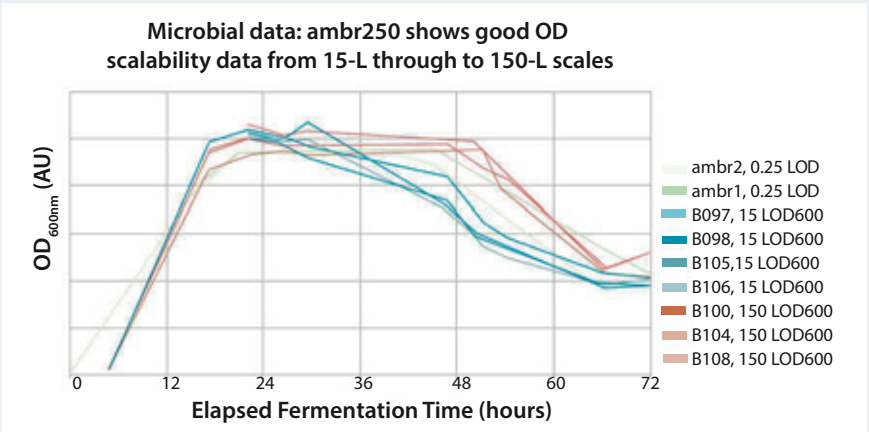
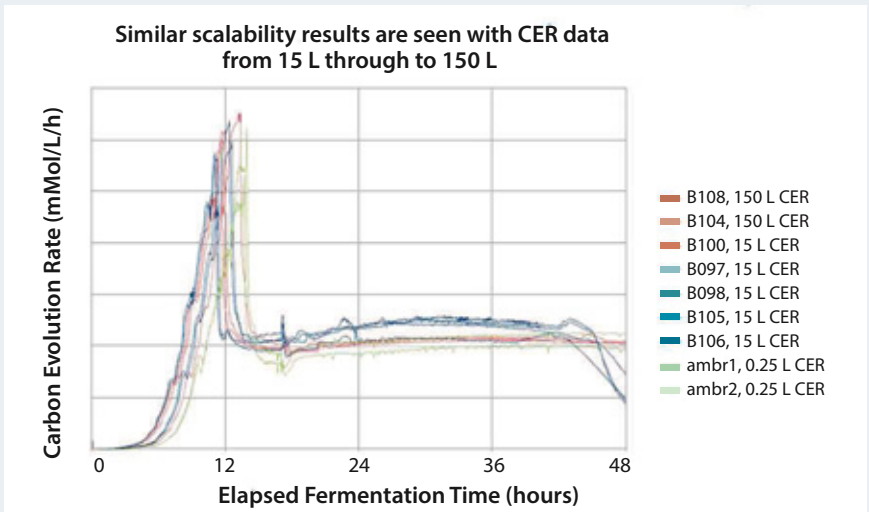


Figure 2: CER profiles of recombinant *E. coli* fermentation in the ambr250 bioreactor system, 15-L and 150-L fermentors



driving biopharmaceutical companies to search for strategies to perform rapid process development for optimizing and scaling-up their manufacturing processes.

Increasingly flat revenues and high development costs have made time-to-market crucial for company profitability. So there is a greater need to release products with desired quality attributes as early as possible. So for biopharmaceuticals, rapid and efficient process development is essential for successful commercialisation (**3**).

One challenge for biologics manufacturers is the burgeoning number of new regulatory requirements. Before a drug or vaccine can be launched, its manufacturer must show that the product meets all regulatory requirements for high quality, safety, and effectiveness. Part of those requirements is to provide robust scientific evidence obtained during process development to support regulatory obligations. As a consequence, there is a need for multifactorial statistically designed research during process development and optimization, leading to a large number of bioprocess experiments, which can be labour intensive, time consuming, and expensive.

Traditionally, technologies such as shake flasks and bench-top bioreactors or spinner flasks for vaccine production have been used for process development. However this approach is manually intensive and prone to human error. It also has a high operating cost and requires a large laboratory.

A well understood biologics manufacturing process is best developed by the application of complex DoE (design of experiment) methodologies. Such methodologies have a very high experimental burden (**4**). All areas of the process must be fully understood, including media components and all process parameters (and the effects of changing them), because such factors alter both titers and product quality attributes.

Because of the inherent problems associated with traditional technologies, the resulting data can include a number of errors that require constant experimental iterations. Such issues lead to costs that are prohibitive to the development of many bioprocessing strategies.

Well-characterized and accelerated process development and optimization can be achieved only if the process can be sufficiently automated in a parallel miniaturised platform that is both easy to set up, scalable, and compatible with disposable technologies. So there is a need to move away from traditional bioprocess equipment and into a platform that enables high-throughput process development and optimization. That type of technology should have three key characteristics:

- miniaturization to enable faster experimental throughput at a low costs
- automation for accurate, reproducible performance of a large number of individual operations
- parallel processing to allow evaluation of a wide experimental space, resulting in process understanding.

Here I present an automated mini bioreactor technology that allows for high-throughput process development and optimization for both microbial fermentations and cell culture processes. In addition, I demonstrate the suitability of this technology by analyzing data obtained from an automated bioreactor and comparing data from laboratory- and pilot-scale bioreactors.

Material and Methods

Mini Bioreactor: The mini bioreactor system chosen for scale-up comparison is the ambr250 automated bioreactor (from TAP Biosystems, a Sartorius Company). This system has three

Figure 3: Growth profile of CHO clones cultured in ambr250 and 3-L bioreactors

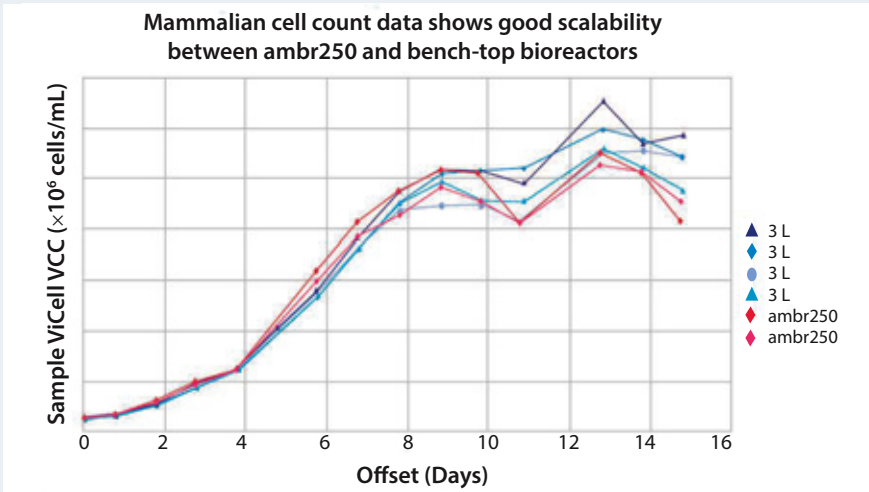
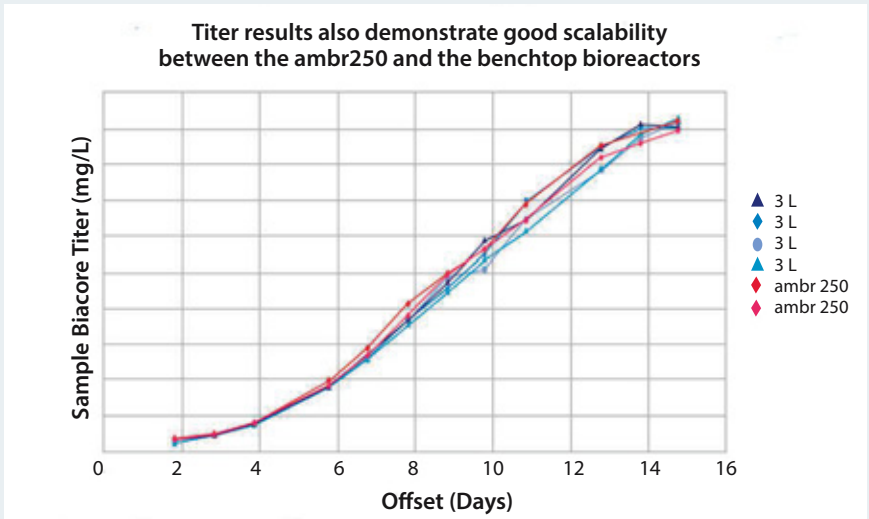


Figure 4: Titre profile of CHO clones cultured in stirred ambr250 and 3-L bioreactors



components: easy-connect single-use 250-mL bioreactors (available in both microbial and mammalian configurations), an automated workstation, and software (Photo 1).

The platform provides increased process volumes, pumped liquid delivery for continuous feeds, and automated individual bioreactor control for all parameters. These features allow a frequent feeding regime and larger volumes to be sampled for performing a wide range of analytical tests. Combined with the parallel control of culture conditions and feeds, such capabilities provide a scale-down bioreactor model that supports quality by design (QbD).

The ambr250 workstation is a class II laminar flow hood that is designed for either a 12 or 24 bioreactor workstation. Both configurations include an automated liquid handler for liquid transfer between bioreactors, sample beds, or media bottles and can be used for automated sampling, inoculation or even media preparation.

Proof-of-Concept

Scale-Up of Microbial Cells: To demonstrate that the ambr250 system is a viable process development and scale-down model for microbial culture, the results from cultures grown in the mini bioreactor, a bench-top fermentor, and a pilot-scale fermentor must be comparable. To this end, recombinant *Escherichia coli* clones expressing a therapeutic protein were chosen as the model organism. The clone cells were cultured in the ambr250 system, which used a single-use microbial bioreactor with a dual 20-mm Rushton impeller. The same clones were cultured in 15-L bench-top fermentors and in 150-L pilot-scale fermentors.

All vessels were inoculated with 2–5% inoculum at an optical density at 600 nm (OD_{600}) between 1 and 5. The cell lines were cultured for 72 hours in chemically defined medium at 37 °C, pH 7.0 ± 0.03, 30% dissolved oxygen (DO), and an impeller speed of gassed power per volume between 5.33×10^2 and 1.43×10^4 W/m^3. Samples were analyzed every 12 hours for cell density using a SpectraMAX plus 384 spectrophotometer (from Molecular Devices) to measure the OD_{600} value of cells.

With the bench-top and pilot-plant fermentors, the carbon dioxide evolution rate (CER) was measured using a Prima PRO process mass spectrometer (from Thermo Scientific). With the ambr250 system, built-in off-gas analyzers measured the CER, with each bioreactor having its own dedicated off-gas analyzer.

Scale-Up of Mammalian Cells: For the ambr250 bioreactor system to be a viable early process development model for mammalian cell culture, the results from cultures grown in it and a in a bench-top bioreactor must be comparable. To show this, recombinant CHO clones expressing a therapeutic antibody were chosen as the model organism. The clones were cultured in the ambr250 system using the single-use mammalian bioreactor and in 3-L bench-top bioreactors. The cell lines were cultured for 16 days in a proprietary, chemically defined medium at 37 °C, pH 7.0 ± 0.3, 40% DO, and with an impeller tip speed of 0.25 m/s. Cells were inoculated at 1×10^6 viable cells/mL, and samples were analyzed every 24 hours using a ViCell cell viability analyzer (from Beckman Coulter). Titres were assessed from day 2 and were then measured every 24 hours using a Biacore 400 analyzer (from GE Healthcare).

Photo 2: The fully automated ambr250 system controls up to 24 × 250-mL bioreactor experiments

Results

Scalability: Optical density and CER profiles of the *E. coli* strains cultured in the ambr250 bioreactor and 15-L and 150-L fermentors showed very similar results (Figures 1 and 2), with the OD_{600} data of the 15-L and 150-L vessels lying either side of the ambr250 data, and CER peaks at the same time points. These results indicate that the ambr250 system provides the capability to be a good scale-down model for process development and optimization of microbial cultures.

The consistent growth and titre profiles of the CHO clones cultured in the ambr250 and the 3-L bioreactor, showed good comparability (Figures 3 and 4) with peak viability, cell densities, and maximum titres at the same time points. The data from the ambr250 system also showed better consistency than did the bench-top bioreactors. This indicates that process development can be reproducibly performed in an automated ambr250 bioreactor system with mammalian cell cultures.

Discussion

Here I outlined a parallel automated stirred mini bioreactor technology to allow for rapid process development and optimization for both microbial and mammalian cultures. Results demonstrated that the ambr250 bioreactor system can replicate larger scales processes (laboratory- and pilot-plant) for both mammalian and microbial cultures, achieving similar results (cell density and titer). This is consistent with published data on CHO, demonstrating that automated mini bioreactors can mimic bench-top bioreactors (**5**, **6**) and shows that an automated mini bioreactor is a comparable model for key parameters such as cell growth and titre in bench-top bioreactors.

The ambr250 bioreactory system enables the automated operation of up to 24 mini bioreactors in parallel, so a full DoE run can be performed in one experiment. This means the study of multiple process parameters is no longer limited by the availability of bench-top bioreactors, operator time, and facility infrastructure. The application of an ambr250 bioreactor system could be used instead of shake-flask and conventional bench-top bioreactors models for process development and optimization. This would save considerable time and resources by reducing manual labour, laboratory support facilities, and large volumes of media.

Because this platform is fully automated, easy to set up, fully disposable, and requires smaller culture volumes, scientists can program and perform high-throughput experiments while achieving highly accurate data. This significantly reduces the need to run repetitive experiments and makes this system a cost-effective tool for process development and optimization of biomanufacturing processes.

The implementation of the ambr250 bioreactor system will improve data quality and allow more complex statistically designed experiments in both mammalian and microbial bioprocess development. This could lead to significantly shorter development timelines and lower costs associated with early stage process development and, ultimately, may contribute to quicker technology transfer and a faster time to market of more affordable biologic drugs and vaccines.

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Single-Use, Stirred-Tank Bioreactors

Efficient Tools for Process Development and Characterization

by Andre Grebe, Christel Fenge, Jean-Francois Chaubard

During the past decade, single-use bioreactors have become widely accepted as an alternative to conventional stainless steel or glass bioreactors for clinical manufacturing and process development. In the biopharmaceutical industry, glass bioreactors are used mainly for process development and optimization, but also scale-down models for process characterization. So it is of significant importance that such vessels replicate the design of production-scale bioreactors for both reusable and single-use applications. Stirred-tank bioreactors with 2-L, 5-L, and 10-L working volumes have proven to be particularly well adopted across the industry. The 2-L version is the work horse of process development, with a volume sufficiently large to serve as a representative small-scale model that allows sampling, yet is easy to handle.

Increasing Effectiveness of Development Staff

Project timelines and workloads can change dramatically, especially during process development. It can be a challenge to keep enough bench-top reactors on hand at all times. Cleaning, setting up, and autoclaving glass vessels requires extra time and effort on the part of laboratory staff, relegating them to regular maintenance rather than performing other, more beneficial tasks. Adding single-use culture vessels that can be used interchangeably with glass vessels to a development laboratory provides significant flexibility, especially during capacity peaks or maintenance periods. That reduces downtime of bioreactor controllers to an absolute minimum.

Photo 1: UniVessel SU bioreactor with optical holder and connection box

Table 1 compares glass and single-use options, estimating a 25% increase of bioreactor controller run time for the latter. Otherwise, additional glass vessels would be necessary to reach equally high use rates; however, they would increase efforts related to change-over activities as well as investment cost. Furthermore, when working with microcarriers, single-use bioreactors eliminate cumbersome and hazardous siliconization. In essence, single-use bench top bioreactors simplify the normal daily life of laboratory staff much like the introduction of single-use shaker flasks did in mammalian cell culture about 20 years ago.

Emulating the Proven Glass Vessel Design

Glass bioreactors have been used for decades and are proven as reliable scale-up and scale-down models of stainless steel and state-of-the-art, larger-scale, single-use, stirred-tank bioreactors. The UniVessel SU single-use culture vessel emulates the design of conventional glass bench-top bioreactors to ensure comparability with previous data generated using such systems. Each unit is delivered irradiated and ready to use right out of the box. It is equipped with noninvasive, single-use pH and dissolved oxygen (DO) sensors to eliminate the need for manipulation of the vessel in a laminar-flow bench to introduce sensors (Photo 1) before initiating a cell culture experiment. Initially, reliability and robustness were sometimes challenging with such systems — especially for pH optochemical patches. But significant improvements of the chemistry and control of single-use patches now ensure reliability and robustness (**1**).

Easily Upgrading Existing Controllers

UniVessel SU vessels can be easily integrated into both new and existing bioreactor control units, reducing investment costs of moving to single-use bench-top systems. Also, the integrated single-use sensor signal can be applied to control pH with a UniVessel SU connection box. It makes cumbersome and risky integration of reusable probes unnecessary. Moreover, the same pH and DO measurement principle can be used as in larger-scale BIOSTAT STR single-use bioreactors that contain the same optochemical probes. Figures 1 and 2 compare classical and single–use sensors with simulated pH and DO step changes — demonstrating good correlation between the probes. Conventional probes can be used in both systems.

Excellent Comparability

Suspension culture of recombinant CHO cells (typically under serum-free or protein-free conditions) is widely used for modern large-scale production of monoclonal antibodies (MAbs) and other therapeutic proteins. A significant body of knowledge has been produced using conventional bench-scale glass culture vessels. So any new product-development or troubleshooting exercise of commercial processes should build on historical data and knowledge, limiting the need to provide supporting data that demonstrate comparability of cell culture systems in use.

Figure 3 shows excellent comparability of the most important process engineering parameter — $k_L a$ and mixing time — of classical glass and UniVessel SU bioreactors at typical impeller tip speeds used in mammalian cell culture (3). In modern MAb production, most companies use platform technologies and robust Chinese hamster ovary (CHO) cell lines. But when dealing with complex, posttranslationally modified recombinant proteins and vaccines, special focus on scale-up and comparability is typically necessary. Especially when cells are grown on microcarriers to produce vaccines, careful consideration must be given to scale-up and comparability of cell culture systems used (4).

Table 1: Comparing single-use and glass bench-top bioreactors

Process Step	Glass Bioreactor	UniVessel SU Bioreactor
Pre- and postrun preparation time (vessel assembly, sensor calibration, autoclaving, medium fill, harvest, cleaning)	2 days	~1 hour
Sterility test	1 day	None
Culture time	12 days	12 days
Possible runs per year per bioreactor controller	24 runs	30 (+ 25%)

Figure 1: Comparing classical and single-use pH measurement in Britton-Robinson buffer solution at 37 °C in the UniVessel SU bioreactor

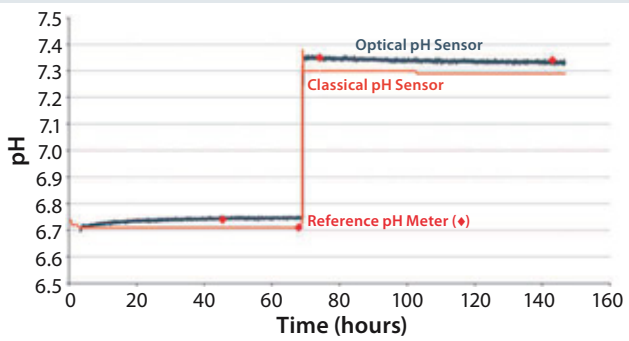
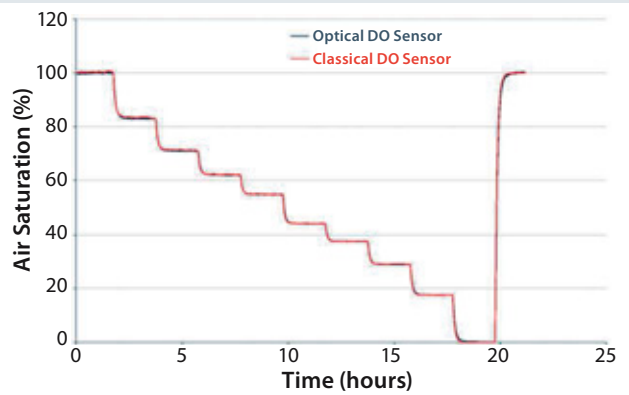


Figure 2: Comparing classical and single-use dissolved oxygen (DO) measurement in reverse-osmosis (RO) water at 37 °C in the UniVessel SU bioreactor



Case Study: A Microcarrier-Based Vaccine Process

A significant number of vaccine processes still rely on adherent cell lines, which require microcarriers as a growth support in stirred-tank bioreactors. This adds complexity to scale-up and scale-down of vaccine processes from the increased shear sensitivity of cells grown on carriers, the larger size of the carriers compared with single suspension cells, and the higher density of carriers. Very often that requires special low-shear impeller designs and careful consideration of the arrangement of probes, dip tubes, and other inserts to prevent formation of dead zones (3). Therefore, bioreactor designs and volumes typically used for suspension culture might be unsuitable as scale-up or -down model systems for microcarrier-based processes. Within the cell and viral technologies department of GlaxoSmithKline Vaccines, special attention is paid to such challenges.

Maintaining comparable process conditions to production scale with regard to shear, homogeneity, and microenvironment for microcarrier-based processes will require particular working volumes and appropriate

Figure 3: Comparing process-engineering parameters of conventional glass and UniVessel SU bioreactors — mixing time (dashed lines) and $k_L a$ (solid lines)

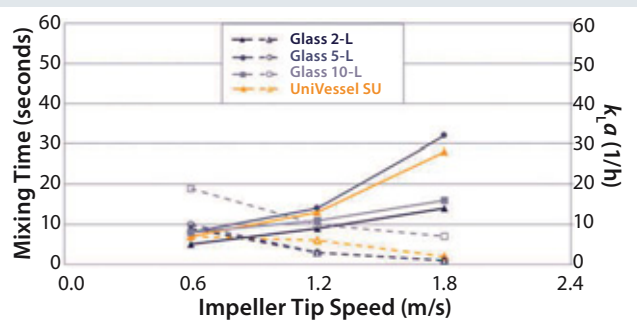


Figure 4: Growth characteristics of an adherent cell line grown on microcarriers in different Sartorius single-use stirred-tank bioreactors (UniVessel SU 2L, BIOSTAT STR 50L, BIOSTAT STR 200L) and the 10-L stirred-tank glass bioreactor typically used as a scale-up/scale-down model

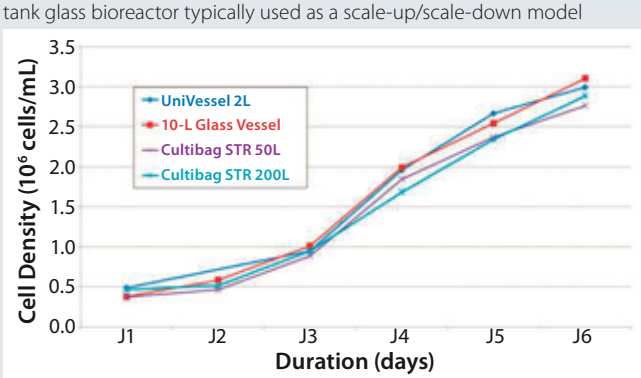


Photo 2: UniVessel SU vessel connected to existing bioreactor controllers at GSK Vaccines (Rixensart, Begium)

designs. In addition, it is often necessary to purify a product so its quality and activity can be determined and process performance can be assessed. Because purification steps are primarily based on product amount, a certain volume is required to achieve required amounts for further purification and product characterization. In this case study, the purification team required a volume of at least 1 L to perform purification experiments.

Taking into account the above-mentioned considerations, the team decided to use bench-top bioreactors of 2–L scale to optimize process parameters. We expected this scale to emulate larger-scale, stirred-tank bioreactor conditions. It would supply the required volume for purification activities, while still allowing for easy and straightforward parallel processing.

To simplify the experimental set-up and increase the throughput of runs per bioreactor controller, the GSK team decided to look for a single-use solution. The team took into consideration the single-use UniVessel SU from Sartorius Stedim Biotech because of its design flexibility and geometrical similarity to classical stirred-tank bioreactors (**2**).

To demonstrate the suitability of the 2-L scale UniVessel SU bioreactor for microcarrier-based processes, the team compared its process performance with that of 10-L stirred-tank glass bioreactors as a benchmark. Those 10-L glass bioreactors have proven to be a representative scale-down model of the GSK larger-scale stainless-steel or single-use (BIOSTAT STR) bioreactors. Figure 4 compares the growth of adherent cells on microcarriers in different single-use bioreactor systems — 2-L UniVessel SU, 50-L and 200-L BIOSTAT STR — with the 10-L glass vessel. Comparable cell growth was obtained in all evaluated stirred-tank bioreactor vessels. Comparable metabolite profiles also were obtained (data not shown), proving the suitability of the UniVessel SU 2-L system for scale-up and -down studies.

Based on comparable process performance and the UniVessel SU system's ease of use, GSK installed a new process-development platform consisting of 12 parallel 2-L single-use, stirred-tank bioreactors (Photo 2). This set-up is primarily used for multivariate design of experiment (DoE) studies, for example that illustrated in Figure 5. It was possible to optimize viral production by evaluating five different process parameters performing only three runs of 12× 2-L single-use vessels demonstrating the effectiveness of such a parallel set-up of single-use vessels.

Thanks to this new single-use process development platform, the team significantly increased the number of experiments that could be run without increasing laboratory personnel. The approach especially helped to further accelerate development timelines and will therefore be extended to other development projects.

Key Benefits

Immediately available, helps to manage peak manufacturing demands

Completely single-use, increases effectiveness of laboratory

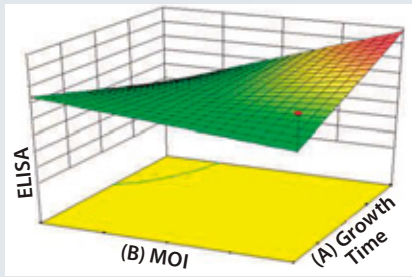
Same design as existing glass vessels, full comparability and scalability

Compatible with existing bioreactor controllers, limited additional investment

Conclusions

We have shown excellent comparability of the single-use 2-L stirred-tank vessel (UniVessel SU 2L) with proven 2-L, 5-L, and 10-L stirred-tank glass vessels regarding $k_L a$ and mixing time. The team also evaluated biological comparability under the most demanding process conditions using a microcarrier-based adherent-cell culture process for viral vaccine production. We could demonstrate excellent comparability of cell growth and metabolite profiles for the single-use 2-L vessel, a 10-L stirred-tank glass vessel historically used as a scale-up/-down model, and larger-scale single-use (BIOSTAT STR 50L and 200L) and stainless-steel stirred-tank bioreactors. Based on these data, GSK established a new process development platform consisting of 12 multiparallel 2-L single-use vessels to support multivariate experimental designs for efficient and fast optimization of vaccine production processes.

Figure 5: Contour plot of a multivariate experimental design to optimize the viral production phase; the response is measured using an ELISA test (ELISA) and shown as a function of infection time (A: growth time) and multiplicity of infection (B: MOI).



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Design of Experiments with Small-Scale Bioreactor Systems

Efficient Bioprocess Development and Optimization

by Andree Ellert and Conny Vikström

Design of experiments (DoE) is one of the most valuable techniques for organized and efficient planning, execution, and statistical evaluation of experiments. Although a DoE investigation can be completed using several runs in one bioreactor, small-scale bioreactor systems designed for parallel operation (such as the ambr15 or ambr250 systems) provide the optimal basis to economically realize a series of experiments. Because of the multitude of interdependent parameters involved in applications such as cell line development, culture media screening, and the optimization of bioreactor operating parameters, DoE evolved as an essential and indispensable method to create process knowledge. It has improved speed of development and has helped define manufacturing processes for high-quality products.

Photo 1: The ambr250 scale-down bioreactor system controls 12 or 24 disposable small-scale bioreactors (100–250 mL working volume) and offers parallel processing and evaluation of multiple experiments while maintaining the characteristics of a larger scale bioreactor.

Design of Experiments: The Efficient Strategy

In pharmaceutical bioprocessing, decreased development time and production costs are key objectives. Within this context, the combined application of process analytical technology (PAT) tools and reliable flexible bioprocess equipment is the basis for an efficient optimization of existing production processes and the development of new ones. Spearheaded by the FDA initiative to use PAT for greater control and process understanding, statistical DoE methods are extensively applied to look for the best process conditions while reducing expensive and time-consuming experiments to a minimum (1).

The concept of DoE is to vary process parameters simultaneously over a set of planned experiments and then interpret the results by means of a proven mathematical model. This model can be used subsequently for interpretation, prediction, and optimization, which allows for greater understanding of processes. By contrast to changing one factor at a time, the DoE procedure delivers optimized information content by using the least number of experiments, thereby reducing development time and labour.

The improvement of production and feed media components is crucial to providing an environment optimal for growth of a used recombinant cell line and formation of a functionally active therapeutic protein (2). Another key target is the optimization of basic process parameters such as temperature, pH, and dissolved oxygen (3). Often, optimization of

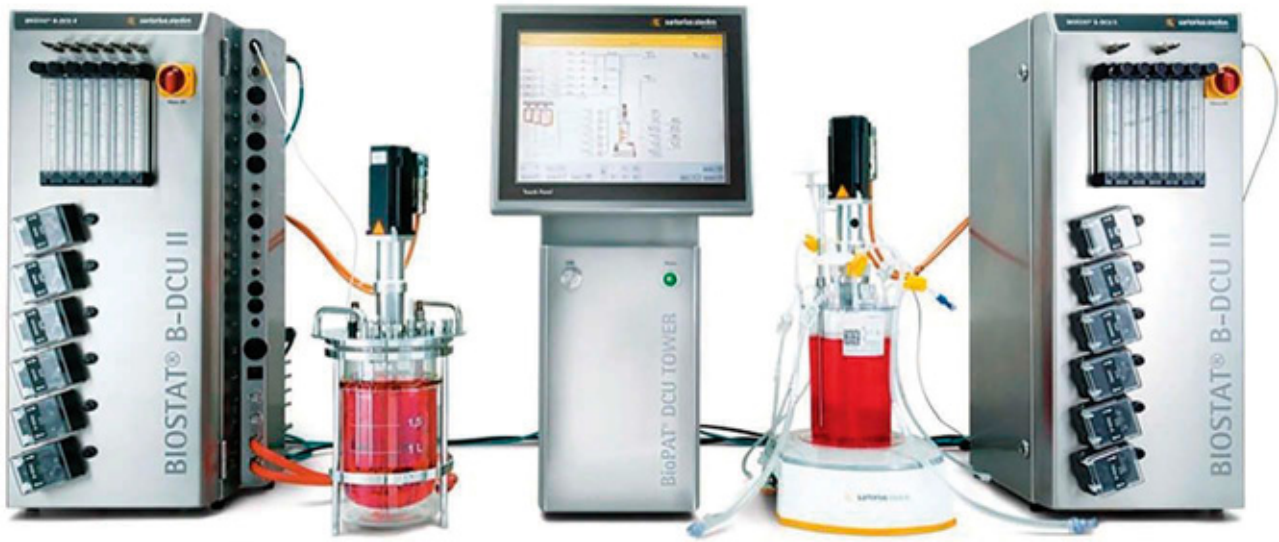


Photo 2: The BIOSTAT B-DCU II system is designed for advanced process optimization and characterization and is available with working volumes of 0.5–10 L; it features independent process control for up to six culture vessels

culture media and state variables are combined, whereas DoE studies can significantly facilitate such tasks (**1**).

Transition from Medium and Feed Development to Process Optimization

Apart from the productivity of a specific cell line, the culture medium has an important impact on both yield and quantity of therapeutic protein and monoclonal antibody produced. The cells require a combination of macro- and micronutrients as sugars, trace salts, vitamins, amino acids, and other components to support cell growth and protein production. Because of the diverse nutritional requirements that are unique to every cell and the large number of medium factors, small-scale systems — with their ability to perform a large number of experiments in parallel — are needed to unlock development bottlenecks.

Historically, media and feed formulations are derived through numerous empirical tests by changing one factor at a time (**4**). This methodology is simple and convenient, but it ignores interactions between components, can miss the optimum completely, and is fairly time-consuming. A superior approach to tackle the problem of media design and shorten the time needed for medium development is to use high-throughput cell culture scale-down systems in combination with statistical DoE approaches.

In addition to improving the medium composition, optimization of the feed-control strategy can be beneficial for fed-batch processes. The practical approach depends on the infrastructure of the bioreactor system itself and the cultivated cell line. Whereas a classical fed-batch with continuous feeding strategies has been proven to be successful for *Escherichia coli* and *Pichia pastoris* cultivations, the method of adding a stepwise bolus of feed solution to a production bioreactor is most widely used in industry for mammalian cell culture because of its simplicity and scalability (**5**, **6**).

The new ambr250 workstation with 12 or 24 single-use stirred tank reactors (TAP Biosystems, a Sartorius company) represents an efficient, high-throughput, scale-down model for initial process development of microbial fermentation or cell culture. The system provides the capability to continuously monitor and control critical parameters in real-time as well as extended feed and sampling options to enable a more effective scale-up and to demonstrate equivalent process performance in comparison to laboratory and pilot scale (**7–9**).

The automated workstation features an integrated liquid handler, which can remove the cap of a single-use vessel to enable initial media load, seeding, sampling, and bolus additions. Culture or feed medium can be transferred preformulated by the pipetting module, or the system can be used to automatically make up media from different components using imported experimental designs. Besides single bolus additions (10 µL to 10 mL), integrated pumps allow continuous feeds of linear or exponential profile ranging from 20 nL/h to 20 mL/h.

Once the most prominent medium components and their optimal ranges are identified, the process can be transferred to a larger laboratory-scale system for experimental verification of the identified optimum to test scalability and to continue with optimizing the bioreactor's operating parameters as well as process characterization studies.

The BIOSTAT B-DCU II benchtop bioreactor (Sartorius Stedim Biotech) is specifically designed for laboratory-scale parallel operations of up to six multiuse or single-use culture vessels with one control tower. It is used widely in the industry for process optimization and characterization (**10**). Each vessel has independent process controls with a wide range of

Figure 1: Comparison of two protein production phases with different factor settings 1 and 2; S_k = fluorescence signal of internal soluble (k = sol) and insoluble (k = IB) protein fraction; θ = cultivation temperature; μ = online observed cell specific growth rate

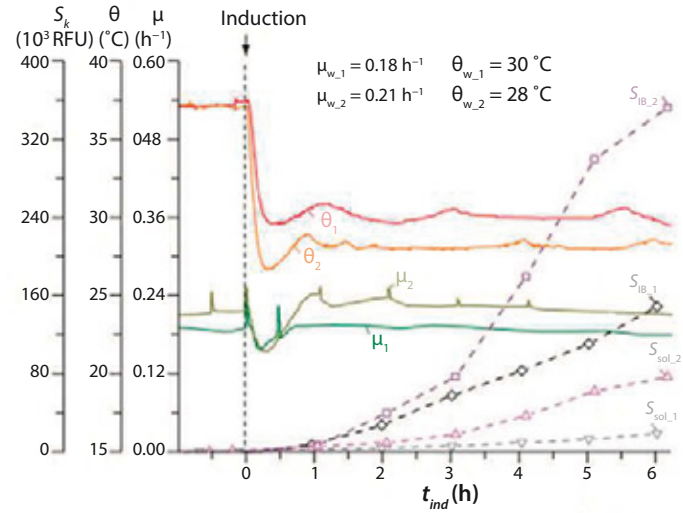
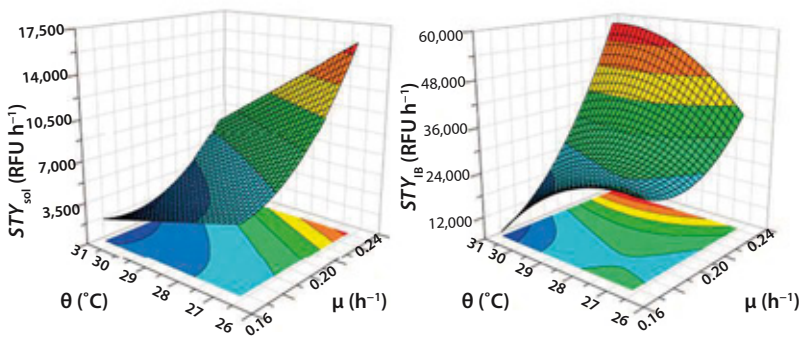


Figure 2: Response surface plots for soluble (LEFT) and insoluble (RIGHT) space-time yield; STY_k = soluble (k = sol) and insoluble (k = IB) space-time yield; θ = cultivation temperature; μ = cell-specific growth rate



measurement and automation features. Systems are preconfigured for immediate use, and they can be used for microbial fermentation or cell culture packages.

Optimization of Recombinant Protein Expression: A Case Study

Within process development divisions, the goal is to identify key parameters that maximize target yield and product quality within process parameter ranges that can be reliably attained at pilot and production scale. In the following case study, a BIOSTAT Q plus six-fold system was used in conjunction with DoE to optimize recombinant protein expression in two *E. coli* BL21 (DE3) strains.

E. coli is one of the most used prokaryotic organisms for the production of active pharmaceutical ingredients (APIs) or parts thereof (e.g., antibody-drug conjugates, ADCs). Testing novel APIs requires the physiologically active form of the protein for preclinical toxicology studies. Although protein solubility does not necessarily correspond to active protein, fast product availability often is limited by low soluble protein yields.

Growth rate, cultivation temperature, and IPTG inducer concentration were investigated for each *E. coli* strain. The effect of each factor and strain on the space–time yield of soluble protein and inclusion bodies was used for process evaluation. Expressed protein was tagged with a green fluorescent protein, allowing for simple and rapid quantification of protein expression with a common fluorescence reader.

For advanced process monitoring and control, the BIOSTAT Q plus system was equipped with O_2 and CO_2 off-gas analysers. Values from those sensors and with the BioPAT MFCS/win bioprocess software allowed for online calculation of cell-specific growth rates as well as data storage and supervisory control. The DoE software BioPAT MODDE was used for experiment definition, statistical evaluation of raw data, and construction of an easy to interpret model.

The Workflow to Success

An experimental design was created to screen for variables that would have the highest effect on the space–time yield. Given the known dependence of growth rate on temperature, the DoE software was used to design a set of experiments with varying levels of each factor. The final experimental plan included four runs on a centred level to determine the variability within the overall system.

Using the BIOSTAT Q plus system, it was possible to screen each *E. coli* strain in as few as 12 runs to identify the most promising strain for further studies. In addition, the inducer concentration was shown to have no significant effect on product yield, so that the lowest investigated concentration could be used for further studies. Once the initial screening procedure was completed, higher levels for growth rate were tested in combination with lower temperature set-points.

Figure 1 shows the optimization potential of the DoE approach while simultaneously varying factor conditions. Using the lowest inducer concentration, different set-points for growth rate (μ_w) and cultivation temperature (θ_w) resulted in a higher soluble and insoluble protein yield for the high/low factor level combination subscripted with 2.

Through this set of optimization experiments and continued interpretation within the DoE software, the process could be further understood finally leading to a substantial increase of soluble protein concentration. Highly predictive

models gave a reliable direction to obtain high space–time yields at low temperature in combination with high growth rate. Figure 2 displays the predicted space–time yields as a response surface spanned by the two factors.

The final test in this experiment was a robustness trial, testing parameters for the maximum allowable process parameter ranges without influencing product quality. Using only six experimental conditions, a safe operating range could be confirmed in which the desired space–time yields were achieved. The design space tools provided by the BioPAT MODDE software can be used to visualize an operating range for the investigated process parameters in a unique probability contour plot considering risk analysis specifications. That guides engineers in determining how likely it is that their experiments will truly identify the most reliable operating parameter ranges.

Conclusions

High-throughput cell-culture scale-down bioreactor systems such as the ambr 15 and the ambr 250 systems can increase efficiency and effectiveness of screening, process development and process characterization exercises. They offer the advantage that a significant number of experiments can be performed simultaneously at small culture volumes. At the same time, the culture environment and bioreactor characteristics of small-scale single-use stirred tank vessels provide excellent comparability to larger scale bioreactors (**7–9**). Leaving the traditional way of trial-and-error optimization behind, advanced DoE approaches enable fast and effective identification of critical process parameters and provide significant cost savings and reduction of development timelines.

Acknowledgments

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Superior Scalability of Single-Use Bioreactors

by Davy De Wilde, Thomas Dreher, Christian Zahnow, Ute Husemann, Gerhard Greller, Thorsten Adams, and Christel Fenge

During the past several years, single-use bioreactors have been gradually established in modern biopharmaceutical processes (**1, 2**). This adoption is directly linked to their unique ability to enhance flexibility and reduce investment and operational costs. Furthermore, production output can be increased, and time to market is shortened (**3**). Today a wide variety of single-use bioreactors exists for the cultivation of mammalian and insect cells (**4**), whereas only limited solutions are available for microbial cultures (**5**).

Typically, processes are established and optimized in stirred-tank benchtop bioreactor systems. One challenge during the development of a robust cell culture process is the straightforward scale-up to final production scale. This is especially critical when using less-characterized bioreactor designs that deviate from the well-known and understood classical stirred-tank principle.

Scale-up is an important and potentially time-consuming step in the development of industrial processes. It involves much more than just doing the same at a larger volume. It requires the generation of solid process understanding at different scales to ensure consistent quality and titer throughout scale-up from early clinical trials to final production scale (**6**). Today, many companies use chemometric tools such as design of experiments and multivariate data analysis to establish critical process parameter ranges that define the design space of a robust production process. Especially during late-phase development of a commercial process, the availability of a properly representative scale-down model of full production scale is essential to allow efficient process development (**7**).

Detailed understanding of bioreactor characteristics at different scales significantly facilitates the development and scale-up of robust production processes (**6**). Typical parameters of concern are oxygen transfer, mixing, and heat-transfer characteristics as well as the generated shear forces.

During the past 30 years, stainless-steel stirred-tank bioreactors have evolved as the gold standard, especially as a result of their straightforward scale-up. Multiple times, their well-understood design principles have proven successful in development and scale-up to safe and robust commercial processes. Furthermore, they enable users to implement their existing knowledge — especially with platform processes — into production processes of new drugs and to set-up experiments in a way that can shorten development timelines.

However, many commercially available single-use bioreactors differ from this gold standard. Vessel design, stirrer design, and gassing strategy especially may differ from the classical

Figure 1: ambr250, UniVessel SU, and BIOSTAT STR family; working volume ranges from 250 mL to 2000 L

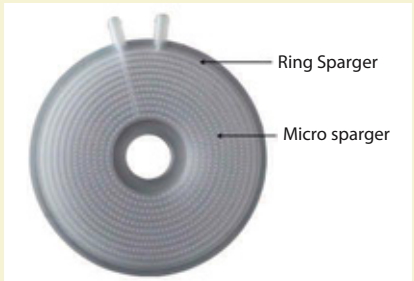
Table 1: Summary of geometrical dimensions of the ambr250, UniVessel SU, and BIOSTAT STR family

	AMBR	UniVessel SU	BIOSTAT STR				
	0.25	2	50	200	500	1000	2000
Total volume (L)	0.36	3	68	280	700	1,300	2,800
Maximum working volume (L)	0.25	2	50	200	500	1,000	2,000
Minimum Working volume (L)	0.06	1	12.5	50	125	250	500
Vessel diameter *D* (min)	62.5	130	370	585	815	997	1,295
Vessel height *H* (mm)	126	240	666	1,055	1,467	1,800	2,330
Ratio *H*/*D*	2	1.8	1.8	1.8	1.8	1.8	1.8
Liquid height h_1 (mm)	90	177	480	783	1,005	1,360	1,670
Ratio h_1/D	1.44	1.36	1.3	1.34	1.23	1.36	1.29
Impeller diameter d_2 (mm)	26	54	143	225	310	379	492
Ratio d_2/D	0.42	0.42	0.38	0.38	0.38	0.38	0.38
Distance between impellers Δ*z* (mm)	30	70	186	300	403	493	640
Size holes ring sparger part (mm)	2	0.5	0.8	0.8	0.8	0.8	0.8
Number holes ring sparger part	1	14	5	25	100	100	200
Size holes micro sparger part (µm)	NA	NA	150	150	150	150	150
Number holes micro sparger part	NA	NA	25	100	500	500	1,000

Table 2: Comparison of process engineering parameters suitable for scale-up from the BIOSTAT STR 50 to the BIOSTAT STR 2000; for scale-up, a CHO process performed at 50 L scale was assumed, which was performed at 150 rpm equivalent to a tip speed of 1.1 m/s, a commonly used tip speed for cell culture applications

Process Engineering Parameter	*N* (rpm)	Tip Speed (m/s)	Re	P/V_L (W/m³)
BIOSTAT STR 50	150	1.1	49,420	22.3
Equal *N* for BIOSTAT STR 2000	150	3.9	605,406	293.1
Equal tip speed for BIOSTAT STR 2000	42	1.1	171,936	6.7
Equal Re for BIOSTAT STR 2000	12	0.31	49,420	0.15
Equal P/V_L for BIOSTAT STR 2000	63	1.6	254,270	22.3

Figure 2: Combisparger for the BIOSTAT STR 2000L system



stirred-tank design principles and do not necessarily offer consistency and geometrical similarity across scales (**8**). So the scale-up exercise might be complicated, and additional risk might be added to the process transfer.

To offer a solution for that, Sartorius Stedim Biotech has developed a range of single-use bioreactors from 250 mL to 2,000 L working volume. Its designs are entirely based on proven stirred-tank bioreactor principles. This ensures straightforward scale-up to 2,000 L scale and beyond to facilitate process transfers to existing legacy production facilities.

Here we detail the different design aspects of those bioreactors and their impact on critical process engineering parameters such as power input per volume, mixing time, and volumetric mass transfer (**9**). We compare the characteristics of the large scale single-use bioreactor family BIOSTAT STR to small-scale single-use bioreactor vessels such as the ambr250 (250 mL) and the single-use UniVessel SU 2L, which are typically used during process development, optimization, and characterization.

Design Principles and Suitable Scale-Up Criteria of Stirred-Tank Bioreactors

Geometrical similarity of vessel design (amongst others defined by the height-to-diameter ratio and the impeller-to-vessel-diameter ratio) is commonly considered important for simple and straightforward scale-up of a process (**10**). This is especially critical as design changes across scales might influence mixing behavior, oxygen transfer, bubble dispersion, and various other key parameters. On the other hand, a homogenous culture environment across scales — where important cultivation parameters such as pH, oxygen partial pressure, temperature, and nutrient supply are well controlled — is a key prerequisite to establish a robust and safe production process. To characterize bioreactor performance across scales and govern scale-up, an appropriate criterion should be defined and kept constant during scale-up. In general, the power input per volume is used as scale-up criterion (**10**). Also, the tip speed or other shear-related parameters are often used, especially when using shear-sensitive cells (**11**) or when growing cells on microcarriers.

Based on the very well-characterized, reusable, stirred-tank bioreactors, it is possible to assign relevant design criteria to single-use bioreactors for animal and microbial cells. One such criterion is the height-to-vessel-diameter ratio (*H*/*D* or aspect ratio), which should be kept within a range of 1:1 to 3:1 for stirred-tank reactors (**10**). A low value for an *H*/*D* ratio results in an increased ratio of headspace surface to filling volume, which enables an

Figure 3: Stirrer speed as a function of the tip speed for the UniVessel SU and BIOSTAT STR reactors.

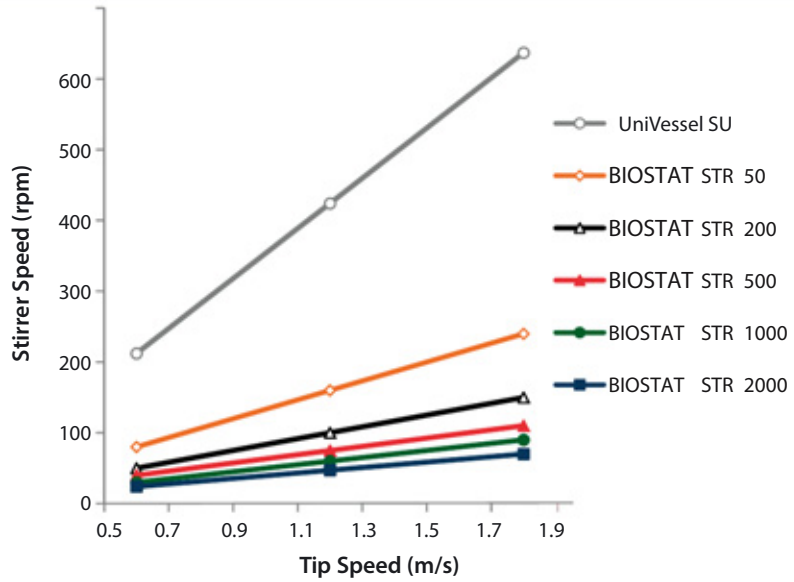
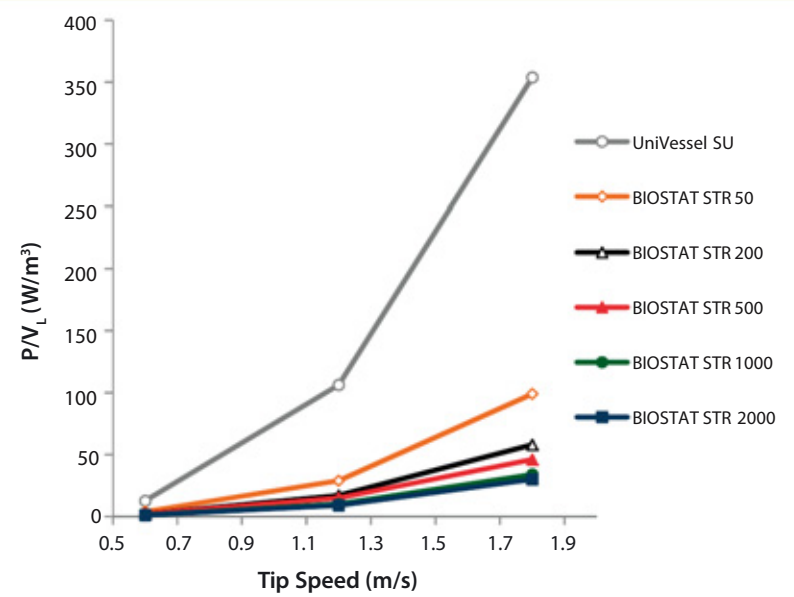


Figure 4: Power input per volume (P/V_L) for the UniVessel® SU and BIOSTAT® STR family



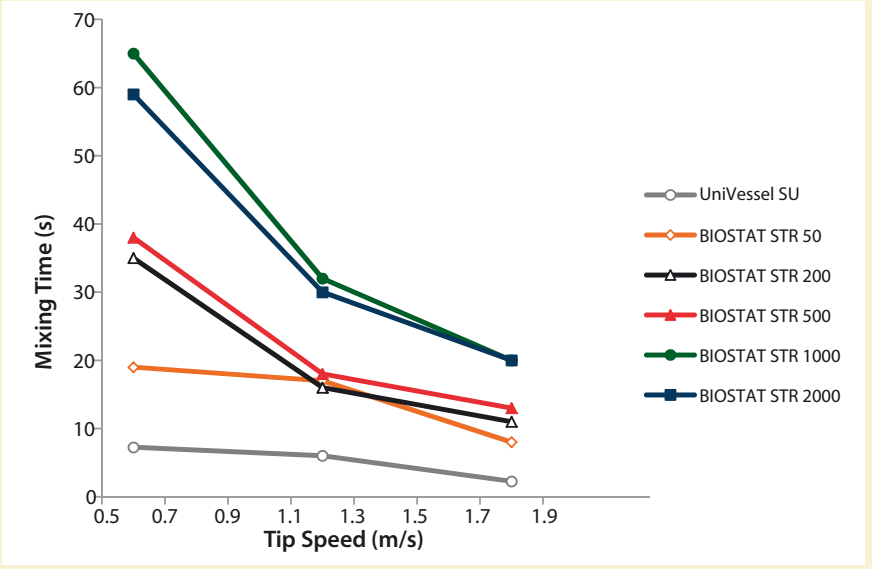
improved gas exchange at the gas–liquid interface. On the other hand, a larger aspect ratio offers advantages in case of direct sparging due to the longer residence time of gas bubbles in the liquid and hence a higher oxygen-transfer rate (**12**). For animal cell cultivations, often a ratio of 2:1 is recommended (**13**).

Another parameter to consider is the ratio of impeller diameter to vessel diameter. This parameter should typically be between 0.33 and 0.5 for animal cells (**14**) and influences mixing efficiency and the generated shear forces. Three-blade-segment impellers or marine-type impellers are commonly used for animal cell cultures (**15**). They efficiently transform the transferred energy into hydrodynamic power and generate large circulation loops because of their axial flow patterns (**16**). Therefore, they are often preferred over Rushton-type disk impellers to achieve effective homogenization. Also, because of the lower dissipative energy transferred, this impeller type is more adequate for shear-sensitive cell lines. In comparison, disk impellers generate a radial flow, leading to higher power input per volume at a given stirrer speed and enhanced gas-bubble dispersion (**11**). It is common to install multiple impellers in bioreactors with an *H/D* ratio above 1:1.4 to ensure efficient mixing throughout the entire cultivation chamber.

To achieve suitable power input, the distance between the impellers is important. It is recommended to use a distance that is 1.2–1.5 times greater than the impeller diameter to guarantee that the impellers act independently of each other. Historically cell culture processes often used two three-blade segment impellers, but today many companies are using a combination of a Rushton-type impeller and a three-blade segment impeller. The disk impeller (installed right above the sparger) ensures good dispersion of the gas bubbles. A three-blade segment impeller serves as a superior axial mixer and ensures homogeneity in the entire vessel, thus supporting high–cell-density processes (**17, 18**). That approach has been facilitated by modern, more robust recombinant cell lines to grow at higher shear rates, which have been selected for commercial manufacturing at large scale (**19**).

Another determining factor for a successful cell culture process is the amount of gas transfer. In conventional bioreactor designs, a sparger is installed directly below the lower impeller, which ensures proper gas-bubble dispersion (**14**). Spargers with small holes generate small bubbles and improve oxygen transfer because of their high gas–liquid interface areas. Reusable bioreactors use sintered stainless steel microspargers. But this design has the disadvantage of nondefined pores, leading to coalescence of bubbles. Therefore, Sartorius Stedim Biotech

Figure 5: Mixing times for the different scales of the UniVessel SU and BIOSTAT STR family as a function of the tip speed for 2 x 3-blade-segment impeller configuration



has developed a special microsparger design with 150 µm holes that provides a uniform bubble swarm of small bubbles for effective gas transfer. Spargers with large holes have a relatively low oxygen transfer but offer improved performance for CO_2 stripping because bigger bubbles typically rise to the gas–liquid interface and carry excessive CO_2 from the cell suspension to the headspace.

At small-scale, CO_2 stripping is less of a challenge. It is more critical at larger volumes because of the higher hydrostatic pressure and thus improved solubility of CO_2. Together with excessive foaming, that limits the efficiency of conventional 10 to 20 µm microspargers for large bioreactor volumes.

Sartorius Stedim Biotech Single-Use Bioreactors Emulate Classical Stirred-Tank Design

Most large-scale, single-use bioreactors do not rely on established design criteria of reusable bioreactors, which can add risk to scaling-up processes. To overcome this, Sartorius Stedim Biotech offers a range of stirred single-use bioreactors (Figure 1) based on classical, well-proven design principles. Different scales exist, allowing to work from 250 mL to 2,000 L culture volumes.

For efficient, cost-conscious process development, highly automated, single-use multiparallel bioreactors are available at 250 mL scale (ambr250) (**20**). This high-throughput process development bioreactor system allows fast and effective establishment of optimal process conditions early in process development. With the classical stirred-tank design, straightforward scale-up is possible either directly for production of material for toxicological studies or through step-wise scale-up through 2 L scale using the UniVessel SU technology and 50 L scale using the BIOSTAT STR. With the UniVessel SU 2 L model, conventional glass vessels can be replaced easily even with already existing bioreactor controllers in development laboratories. The UniVessel SU design is similar to its glass counterpart and the large-scale single-use BIOSTAT STR design, thereby enabling straightforward scale-up all the way from development to commercial production and offering a single-use scale-down model for process characterization.

The BIOSTAT STR has a cylindrical shape with an *H/D* ratio of 2:1 and a semi-torispherical bottom and top. The impeller-to-bag-diameter-ratio is 0.38 with a distance between both impellers of 1.3× the impeller diameter. So the vessel design fits perfectly to the gold standard derived from reusable bioreactors for mammalian cell culture. Table 1 provides a detailed description of the geometrical dimensions of those closely linked single-use bioreactor families.

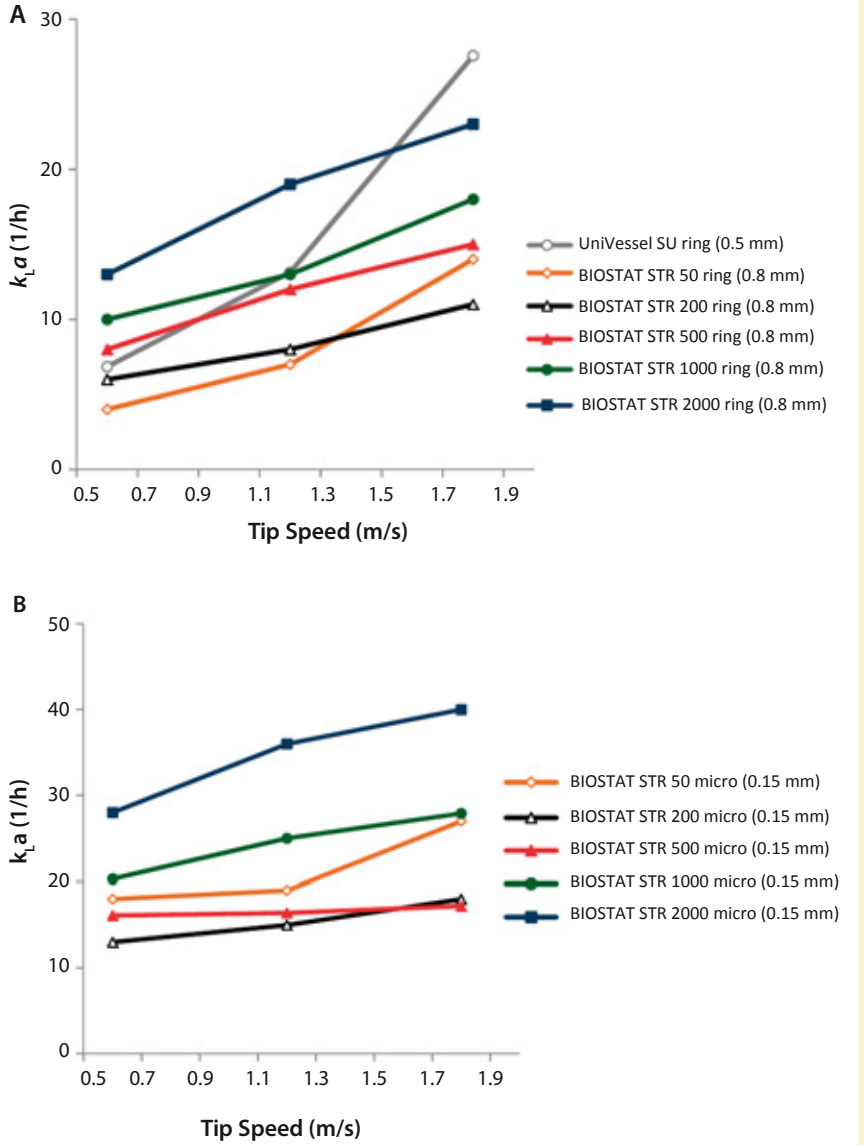
The impellers are installed on a rigid, central shaft. For agitation, 2 × 3-blade-segment impellers are available. For the BIOSTAT STR, a combination of a six-blade-disk (bottom) and three-blade-segment (top) impeller can be installed as an alternative.

The study presented here focuses on process engineering characterization of a configuration based on 2 × 3-blade-segment impellers for different single-use bioreactor volumes. Gas transfer has been characterized for a microsparger (hole diameter = 150 µm) or a ringsparger (hole diameter = 0.5 mm for the Univessel SU or 0.8 mm for the BIOSTAT STR) positioned below the lower impeller. The BIOSTAT STR is available with a combisparger (Figure 2) — consisting of both a microsparger (0.15 mm holes) and a ringsparger (0.8 mm holes). The microsparger supports high oxygen transfer, and the ringsparger enhances CO_2 stripping. All single-use bioreactors from 250 mL scale to 2,000 L are equipped with precalibrated, single-use optochemical probes for pH and pO_2 measurement. Alternatively, conventional probes can be introduced if desired for scales ≥2 L. All single-use bioreactors are available with standard digital control units.

Process Engineering Characterization

We performed in-house process engineering characterization of the UniVessel SU and BIOSTAT STR bioreactors. For the ambr250 system, we used data published by Bareither et al. (**20**). The process engineering characterization of the UniVessel SU and BIOSTAT STR family was performed at parameters typical for mammalian cell culture (tip speeds between 0.6–1.8 m/s). For the characterization of the ambr250 Bareither et al. used tip speeds

Figure 6: Characteristics of the volumetric mass transfer for the different scales of the UniVessel SU and BIOSTAT STR family using 2 × 3-blade-segment impellers; (A) results of the ringsparger and (B) results of the microsparger



ranging from 0.27 m/s to 1.02 m/s, which resulted in corresponding stirrer speeds from 200 to 800 rpm (**20**).

Figure 3 graphs stirrer speed as a function of tip speed for the UniVessel SU and BIOSTAT STR systems. The increasing impeller diameters requires lower stirrer speeds at increasing scale to maintain the same tip speed.

During in-house trials for the BIOSTAT STR and UniVessel SU bioreactors, the Newton number (Ne) was determined to characterize the different impeller types and configurations and to quantify the power input per volume. The Newton number was determined by torque measurements (**8**). For 2 × 3-blade segment impellers, we calculated a Ne of ~1.3. The fact that the Reynolds number (Re) is above 10,000 at the chosen tip-speed range implies that turbulent flow conditions are present, thus the Ne value is constant.

The power input per volume (P/V_L) is an important process engineering parameter and can be calculated based on the experimentally determined Newton number. Figure 4 shows the power input per volume for the BIOSTAT STR and the UniVessel SU bioreactors. For the ambr system, Bareither et al. (**20**) reports a power input of 10–445 W/m^3 for the tip speed. To maintain a constant P/V_L value during scale-up, the tip speed will need to increase with increasing scale. That clearly demonstrates that users have to choose their scale-up criterion because it is not possible to keep both the tip speed and the power input per volume constant when increasing the bioreactor scale.

We determined mixing times for the BIOSTAT STR and UniVessel SU bioreactors using the decolorization method (**14**) to characterize the mixing capabilities of the single-use bioreactor systems. It is obvious that the mixing time decreases with increasing tip speed because of the higher power input per volume. In addition, we obtained higher mixing times with increasing scales.

Figure 5 shows the mixing efficiency for the different single-use bioreactor scales. At 2 L scale, a mixing time of 7 s at a tip speed of 0.6 m/s and 2 s at a tip speed of 1.8 m/s can be achieved. At 50 L scale, mixing times increase to 19 s at tip speed of 0.6 m/s and 8 s at a tip speed of 1.8 m/s. For 2,000 L scale, mixing times of 20 s can be obtained. Overall, it is possible to ensure mixing times <30 s for all scales, from 2 L to 2,000 L, thus demonstrating the superior performance of the UniVessel SU and BIOSTAT STR family for mammalian cell culture (**9**).

For the quantification of the oxygen-transfer rate, we determined the volumetric mass transfer coefficient (k_La) using the gassing out method (**21**). For most common cell cultures, k_La values of 5–10 h^{-1} are required. Figure 6 shows the k_La characteristics for different tip speeds and scales of the BIOSTAT STR and UniVessel SU bioreactors. Both ring and microsparger elements were used for the BIOSTAT STR system and a ringsparger for the UniVessel SU and ambr studies.

The k_La-values increase with increasing tip speed at any given scale and configuration. As expected, the k_La values increase also with the scale. That can be explained by the increased liquid height and the longer residence time of the gas bubbles in the liquid — resulting in a more efficient oxygen

transfer (**22**). The ambr system had $k_L a$ values of 8.5 h^{-1} at a tip speed of 1.02 m/s (**20**). For the other bioreactor families $k_L a$ values >10 h^{-1} can be easily achieved at all scales for both microsparger and ringsparger. Hence, the ambr, UniVessel SU, and BIOSTAT STR bioreactors meet the oxygen-transfer requirements of mammalian cell cultures. With a microsparger, $k_L a$ values up to 40 h^{-1} can be reached at 2,000 L scale, thereby demonstrating the superior performance of this bioreactor type and making this bioreactor type the ideal choice for high cell-density processes.

Superior Single-Use Bioreactor Scalability Due to Classical Stirred Tank Principles

Their excellent performance characteristics make the single-use ambr250, UniVessel SU, and BIOSTAT STR bioreactors ideal for mammalian cell culture — even for very demanding, high cell-density or microcarrier-based processes. Low-shear agitation with three-blade segment impellers provides homogeneous mixing. With a nonparticle-shedding microsparger, operators can reach oxygen transfer rates of up to 40 h^{-1} at 2,000 L scale. Like other single-use bioreactors, such systems make cell culture operations more flexible, cost-effective, and less time-consuming. Their classical stirred-tank design allows relying on well-known and established scale-up criteria such as power input per volume or tip speed. In addition, their geometrical similarities across all scales facilitate successful scale up and scale down as well as process transfer, thus derisking scale-up and process transfers significantly. These single-use stirred tank bioreactor solutions enable seamless scale-up from 250 mL to 2,000 L. This makes them ideal solutions for any modern biopharmaceutical development and production facility for monoclonal antibodies, recombinant proteins, and vaccines.

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Integrated Optical Single-Use Sensors

Moving Toward a True Single-Use Factory for Biologics and Vaccine Production

by Henry Weichert, Julia Lueders, Joerg Weyand, Thorsten Adams, and Mario Becker

Through the past decade, single-use bioreactors for culturing mammalian and insect cells have been widely adopted in preclinical, clinical, and production-scale biopharmaceutical facilities (**1**, **2**). With such bioreactors in operation, monitoring and control of process parameters is vital for ensuring critical quality attributes (CQAs) of biologicals or vaccines are met for production of a safe product. Traditionally, bag-based and bench-top vessels have been fitted with conventional pH and dissolved oxygen (DO) probes similar to those used in stainless steel or bench-top bioreactors. DO and pH are the most commonly monitored physicochemical parameters measured in real time. For many processes, pH measurement and control is critical because small deviations can influence culture growth and metabolism, particularly glucose consumption and lactate production (**3**). DO must be monitored and controlled to prevent changes in oxygen concentration, which can cause problems such as excessive lactate production, reduced antibody glycosylation, or cytotoxicity (**4**, **5**).

Reusable sensors usually are calibrated separately, mounted in probe assemblies, autoclaved, and then fitted by means of an aseptic connector to a single-use bag. Such time-consuming manual tasks can reduce many benefits of single-use bioreactors and could even introduce contamination.

Photo 1: Different scales of single-use bioreactors with integrated, single-use pH and DO sensors; (LEFT TO RIGHT) UniVessel SU, BIOSTAT RM 20, and BIOSTAT STR 2000 systems from Sartorius Stedim Biotech

Single-use sensors offer a number of advantages to scientists using disposable bioreactors: lessening contamination risks and eliminating cleaning, associated validation, sterilization, and probe-calibration steps. Despite the benefits of these sensors, the biopharmaceutical industry has been slower to adopt them than single-use bioreactors. One reason for that was stability issues related to irradiation, drift, and sensor lifetime of optical pH sensors compared with traditional probes. Also, few single-use bioreactors come ready equipped with these sensor types at different scales.

Here, we describe Sartorius Stedim Biotech's integrated single-use sensor assemblies for different scales of single-use bioreactors. They are designed to ensure robust performance and be comparable to conventional probes. Case studies show that in real large-scale cell culture runs up to 1,000-L scale. Advances in single-use sensor design and performance could enable the biopharmaceutical industry to move closer to completely single-use production of biologicals of consistent quality.

Integrated Design

For integration into single-use bioreactors, optical single-use sensors must be designed so that they can be fitted to single-use bags or containers and irradiated. Then they have to be ready to calibrate easily and function accurately after irradiation in real-time cell culture conditions. Optical sensors designed for integration into single-use bioreactors described herein determine pH and DO concentration

Table 1: Technical features of optical single-use pH and DO sensors (from PreSens Precision Sensing GmbH) used in Sartorius single-use bioreactors.

	Single-Use pH Sensor	Single-Use DO Sensor
Patch composition	Polymer matrix with two linked dyes	Silicon matrix with integrated dye
Dyes	pH-sensitive fluorescein derivate with a nanoparticle encapsulated reference dye	Platinum derivate
Wavelengths	Excitation at 480 nm, emission at 570 nm	Excitation at 505 nm, emission at 630 nm
Measurement method	Dual lifetime referencing method (DLR): This method enables internally referenced measurements. A combination of different fluorescent dyes detects intensity changes in the time domain.	Fluorescence quenching: Light from a blue-green LED excites an oxygen sensor to emit fluorescence. If the sensor spot encounters an oxygen molecule, excess energy is transferred to that molecule in a nonradiative transfer, decreasing or quenching the fluorescence signal.
Calculation of pH or DO values	Based on existing pH, a pH-sensitive dye undergoes a phase shift, which is compared with the reference dye. Final pH is calculated according to the Boltzmann equation and a calibration curve.	Dye emission depends on O_2 concentration. O_2 quenches a fluorophore and reduces light emission. Final values are calculated using the Stern–Volmer equation, applying calibration parameters as constants.
Measurement ranges	pH 6–8	0–110% at 37 °C

based on fluorescence and luminescence principles, which are well-established methods of determining those parameters for cell culture (**6**, **7**). When fitted to a single-use bioreactor, these sensors are connected to a measurement amplifier that transmits light of a specific wavelength to a sensor patch. Fluorescent dyes in that patch are stimulated by sinusoidally modulated light, allowing pH or O_2-dependent emission intensity to be captured by a phase shift from an excitation to a fluorescent signal (**8**).

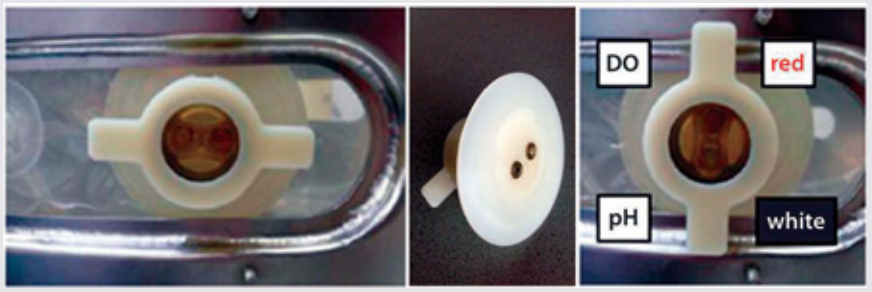
Table 1 summarizes the main features of optical single-use DO and pH probes (from PreSens Precision Sensing GmbH) that are used in Sartorius Stedim single-use bioreactors. DO and pH patches are fixed on the inner surface of a single-use bag or container, allowing both parameters to be measured noninvasively from outside through the polymer wall. That eliminates contamination risk because the sensors are introduced to the bioreactor assembly before sterilization. The single-use DO sensor offers additional advantages over traditional, reusable, glass Clark electrodes because it requires no electrolytes. That significantly reduces equilibration times, which saves time in use by making the integrated single-use bioreactor and sensor ready to calibrate with cell culture media at the point of installation.

Scalability

For seamless scalability of biologics and vaccine production processes, single-use sensors need to be integrated into at laboratory-, pilot-, and manufacturing-scale bioreactors. That reduces the risk of differences in cell culture parameters that can come with different measurement principles. It enables process development and biologics production with consistent CQAs at each scale throughout a product's life-cycle, so processes can be scaled and transferred without needing time-consuming additional investigations.

Sensor patches often have to be located in different places and connected up differently for optimal measurement of pH and DO, depending on the bioreactor design and materials and/or the type of cell culture mixing used (Photo 1). For example, on a rigid, stirred, 2-L single-use UniVessel SU bioreactor, single-use sensor patches are integrated into the vessel bottom, and read-out comes directly through free-beam optoelectronics. In the rocking motion BIOSTAT RM system, sensors are installed by means of tubes and their read-out comes through a flexible optical fiber cable. On a BIOSTAT STR bioreactor, sensors are integrated into sensor ports that protect them during gamma irradiation. All BIOSTAT STR bags can be equipped with a second pair of sensors to provide back-up probes because such bioreactors are designed for good manufacturing practice (GMP) production.

Photo 2: Sensor fitted to stirred, single-use bioreactor with sensor port in "closed" (LEFT) air-protected position and "open" position (RIGHT) for cell culture use



Preparation of Integrated Single-Use Sensors

Sterilization: To produce fully integrated, single-use bioreactors and sensors that are ready-to-use and consistent in quality, the assembly must be provided as one sterile unit. Bioprocess scientists then can begin using such integrated bioreactors without having to fit sensors, which can introduce the possibility of contamination.

Gamma and beta irradiation are established methods of sterilizing single-use bioreactors. However, single-use pH sensors can be affected during irradiation by acidic gases and ozone generated by gamma or beta

Figure 1: Calibration curves of single-use pH sensors protected from residual air by the sensor port and subjected to different irradiation doses

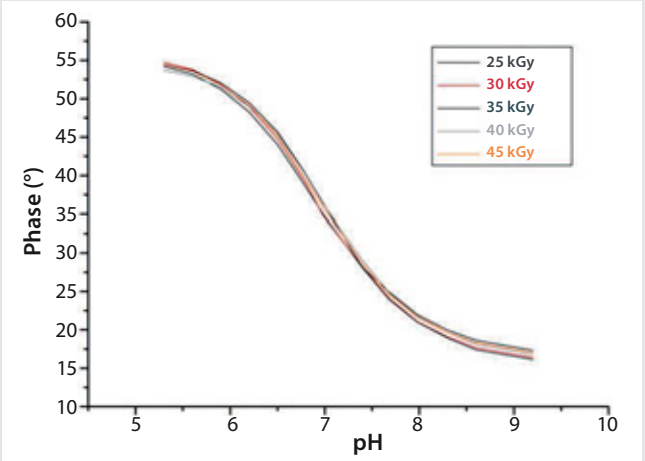
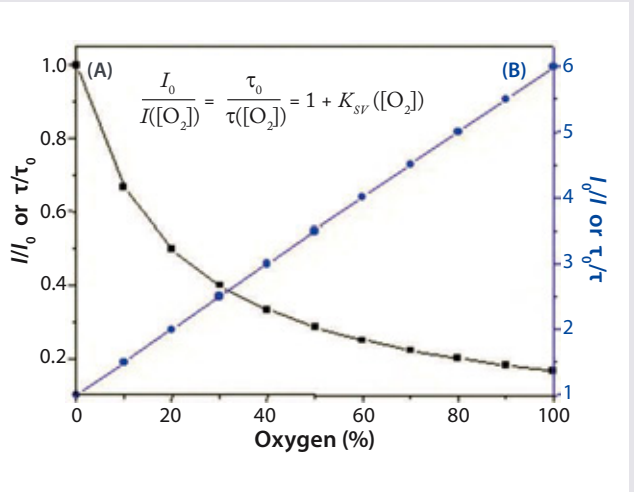


Figure 3: Calibration function of optical DO sensors

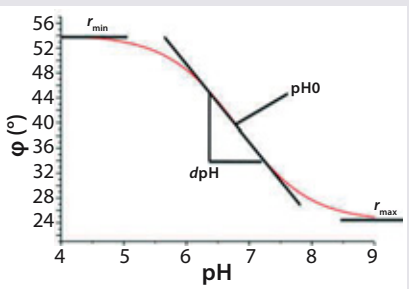


beams in the surrounding air. That may cause a loss of active dyes in sensor patches and impair measurement performance, especially for pH dyes. When producing integrated bioreactor and pH-sensor assemblies, methods are needed to protect sensor patches from residual air in those assemblies. For example, BIOSTAT STR stirred systems contain internal stirrers and spargers, which prevent bags from folding flat and thus create air pockets.

A highly specialized design of sensor port protects sensors during irradiation of such containers (Photo 2). It has a closed (irradiation, storage) position that isolates sensors from the air and a working position in which they come into contact with the bag lumen. By contrast, rocking-motion single-use bioreactors can be flattened to contain marginal residual air volumes, making no additional measures necessary to protect them during irradiation. Similar to such bioreactors, the residual air volume in UniVessel SU vessels is small, so optical sensors remain unaffected. Also, UniVessel SU bioreactors are beta irradiated, which is a gentler type of sterilization.

Gamma irradiation does not affect single-use pH probe performance alone. Figure 1 demonstrates reproducible and consistent pH measurements of optical pH sensors that have been subjected to a broad range of irradiation doses. The data show that it is possible to sterilize a single-use sensor and bioreactor together and provide a fully sterile and functional assembly, which is ready to use from installation.

Figure 2: Calibration function of optical pH sensors



Calibration: From each batch of ~1,000 patches, a defined number of sensors are used to generate calibration values after irradiation. This is part of routine quality control (QC) and ensures that sensors in each batch meet stringent quality acceptance criteria. An integrated quality management system ensures full traceability of all production steps, including raw-material lots, intermediates, and sensor components.

Calibration of single-use PreSens pH sensors involves four calibration parameters approximated from the sigmoidal calibration functions: r_{min}, r_{max}, dpH, and pH_0 extrapolated by a curve fit according to the Boltzmann equation (Figure 2). For calibration of a single-use PreSens DO sensor, the correlation between oxygen concentration and luminescence lifetime is expressed by the Stern–Volmer equation (Figure 3). Because oxygen and luminescence are directly proportional, only two points measured at 100% and 0% air saturation are necessary for calibration.

To calibrate a single-use pH sensor for specific cell culture process conditions, a one-point calibration is necessary after the medium reaches a temperature and CO_2 equilibrium. This must be performed because of differences between cell culture media and reference systems, the latter having been used to determine calibration parameters. We recommend daily off-line sampling for pH measurement to determine whether deviations go beyond defined criteria, typically 0.1 pH units. If it has done so, then a single-use pH sensor should be recalibrated.

A recalibration is also necessary when the ionic strength of a medium is altered (e.g., through addition of base to control pH). Figure 4 demonstrates a calibration-curve shift caused by changes in ionic strength over a range of 50 mM to 200 mM, which represents the most common range for mammalian cell culture processes and related feeds.

Ionic strength represents electric field strength based on the total amount of ions in solution. They influence conductivity of electrolytes, including the electrolyte gel in optical patches. By comparison, osmolarity measures the total amount of osmotic active substances in solution, representing not only ions, but also carbohydrates and proteins, which do not influence ionic strength of a medium.

Figure 4: Calibration-curve effect of changing in ionic strength

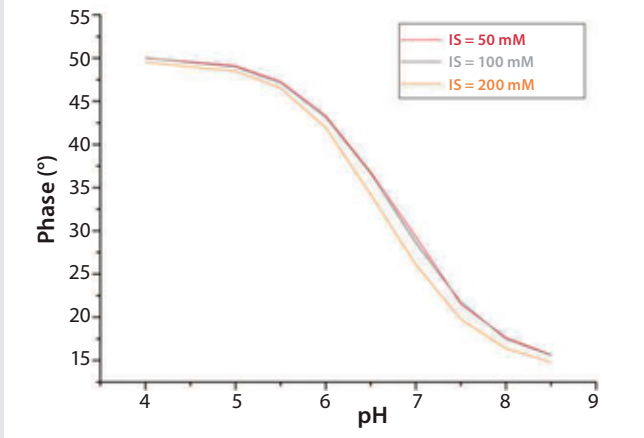


Figure 5: Calibration curves after accelerated aging of single-use pH sensors up to three years after gamma irradiation

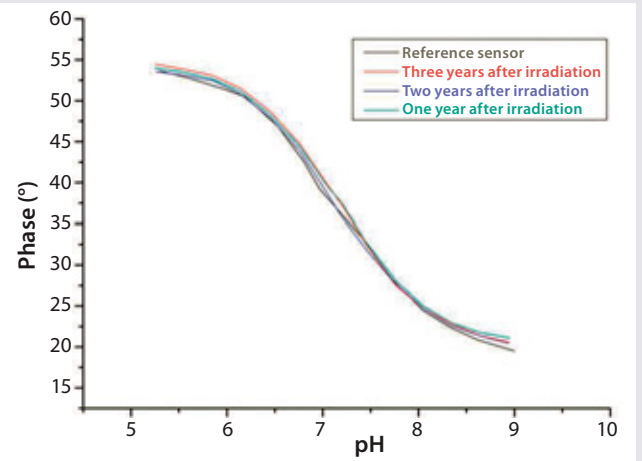


Figure 6: Continuous pH measurement by a single-use optical sensor and classical pH probes in buffer at two different pH values

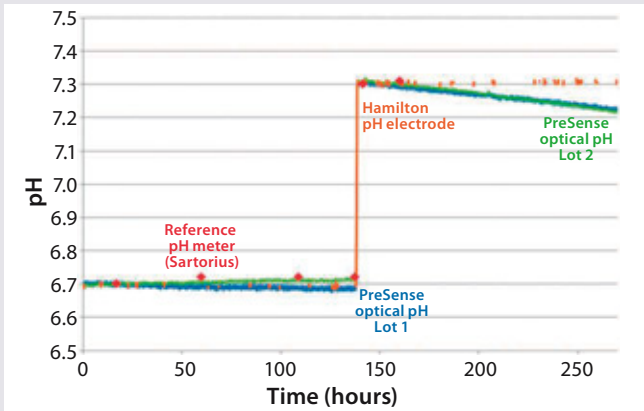
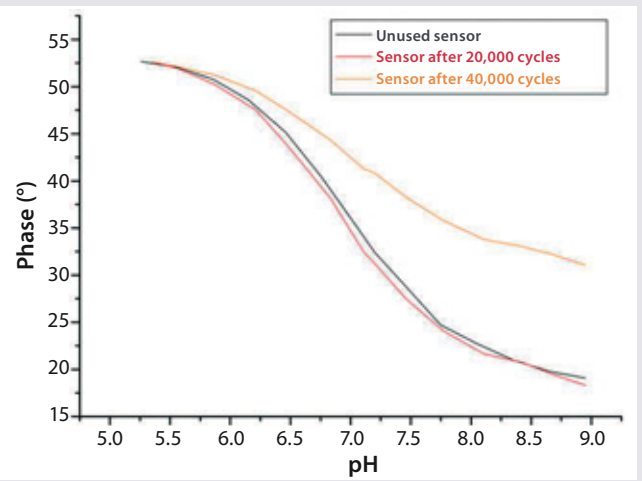


Figure 7: Calibration curves of used pH sensors compared to unused sensors; good comparability is achieved between a sensor exposed to 20.000 measuring cycles and an unused sensor.



Operational Considerations: Fluorescence dyes in optical sensors are sensitive to light. Exposure to intense light can cause irreversible damage to those sensors, making them inoperable. So bioreactors with integrated optical sensors must be stored in light-proof packaging until bioprocess runs begin to keep the effect of light to a minimum established for all Sartorius Stedim Biotech single-use containments. And during operation, users should minimize direct exposure of optical sensors to light sources.

Stability: The shelf life of single-use sensors has been established at two years before and up to three years after gamma irradiation. So they can be stored for up to two years before being assembled into an integrated single-use bioreactor, which then has a shelf life of two years after irradiation. The single-use sensors will maintain their calibration parameters within strict acceptance criteria even for three years (Figure 5), so they are no longer the limiting factor for shelf life of single-use bioreactors.

Case Study: Long-Term Stability in Buffer System

As part of implementing optical sensors into different single-use bioreactors, we executed detailed performance tests. The first test measured pH every minute for >11 days at 37 °C with a buffer system at an ionic strength 150 mM (Figure 6). The pH value was controlled using classical pH probes (Hamilton easyferm), with reference pH measured using a Sartorius bench-top pH meter. That pH probe was kept in potassium chloride (3M KCl) and calibrated before every single measurement.

After 140 hours, we shifted the pH from 6.7 to 7.3 using 1 molar sodium hydroxide (1M NaOH) and recalibrated the optical single-use sensors. Deviation between the optical single-use pH sensor and the reference pH probe was <0.03 pH units for about 200 hours. That equates to ~12.000 single measurement points and <0.1 pH units for about 270 hours, ~16.000 single measurement points. This test demonstrated long-term stability and accuracy of the single-use pH sensors compared with conventional bench-top and inline pH probes with a buffer system.

Case Study: Cell Culture

In cell culture, pH control is important to maintaining optimum growth and metabolism. Frequent pH measurement is not required with Chinese hamster ovary (CHO) and other mammalian cell lines because their growth and metabolism are slower than those of microbial cultures, with typically simple step changes in pH involved. So a measuring frequency of once per minute is again effective to provide sufficient data for robust control loops.

Figure 8: Measurement of pH by a single-use, optical pH sensor and a classical online and offline pH probes of a 50-L fed-batch CHO cell culture grown in a BIOSTAT STR 50 bioreactor; the single-use pH sensor was recalibrated daily.

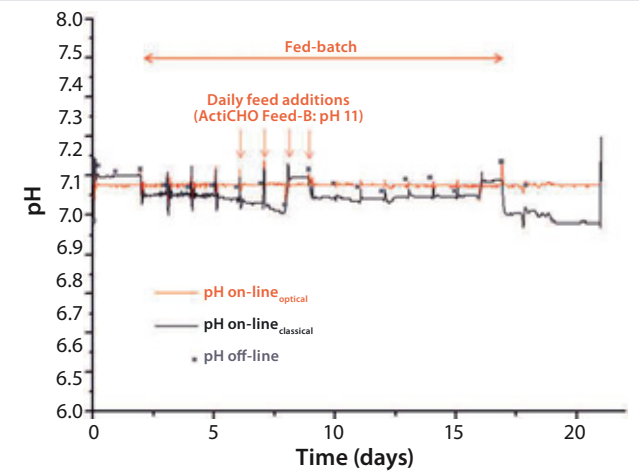
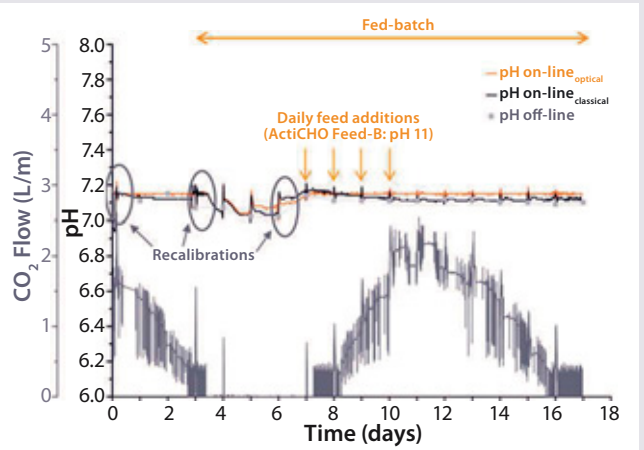


Figure 9: Measurement of pH by a single-use, optical sensor and classical on-line and off-line pH probes of a 1,000-L fed-batch CHO cell culture; the single-use sensor was recalibrated on days 1, 3, and 6.



Optical pH sensors have a life time of ≥20,000 read-outs. At that measuring frequency, continuous measurement over 14 days is possible without any significant loss of performance during cultivation (Figure 7).

Single-use pH sensors integrated into BIOSTAT STR 50 and 1000 bags provide comparable results to on-line and off-line pH probes, as shown with 50-L and 1,000-L fed-batch CHO cell cultures over 17 days (Figures 8 and 9). Our results demonstrate that integrated single-use pH sensors are reliable and robust and provide a scalable method for monitoring pH during a full cell culture run.

Conclusion

In bioprocess applications, single-use sensors provide consistent and precise measurement that compare well to traditional online and offline probes. Single-use bioreactors with integrated optical single-use sensors are available at different scales (from 15 mL to 2,000 L). The sterilization and design of optical probes have been optimized for the type of single-use bioreactor to which they will be fitted. That allows for robust measurement performance throughout each cell culture run. The sensors provide process development scientists with good data comparability across scales: from small-scale rigid vessels used in process development and characterization all the way to 2,000-L bag-based bioreactors for clinical and commercial production (including seed-train bioreactors such as rocking-motion systems).

Easy and straightforward single-use sensor operation and calibration provides accurate data as long as optical sensors are protected from direct light. Such sensors are integrated into single-use bioreactors to create a single, sterile, sealed unit. That allows for rapid set-up with easy alignment with automated control systems and prevents bioreactor contamination by requiring no fitting of reusable probes.

Development of robust, integrated, single-use sensors and bioreactors thus enables the biopharmaceutical industry to move closer to implementing automated, fully single-use factories to produce safe and effective biologicals and vaccines.

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Diatomaceous Earth Filtration

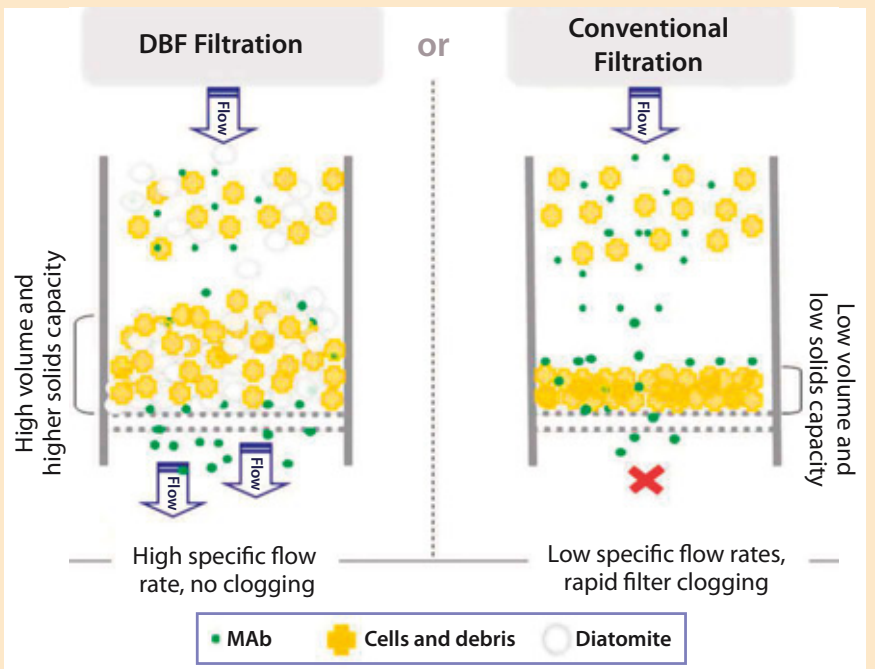
Innovative Single-Use Concepts for Clarification of High-Density Mammalian Cell Cultures

by Tjebbe van der Meer, Benjamin Minow, Bertille Lagrange, Franziska Krumbein, and Francois Rolin

In the past decade, biopharmaceutical manufacturers have demonstrated major improvements in monoclonal antibody (MAb) production, exhibiting product titers frequently in the range of 5–10 g/L using standard fed-batch mammalian cell cultures (**1**, **2**). Increased product yields allow for smaller-scale production vessels. With 2,000-L single-use bioreactors already commercially available, single-use manufacturing of biomolecules becomes more and more an option. Other recent developments in the biopharmaceutical industry — e.g., drugs for smaller indications and more potent drugs allowing for lower dosages — will further stimulate the demand for smaller and more flexible single-use manufacturing facilities.

Although single-use technology in general has matured considerably over the past few years, some unit operations (e.g., cell removal) still need more attention to become more economical and robust. High product titers often result from increased cell densities rather than increased specific productivities per cell, and the resulting solids content poses considerable challenges on commonly applied harvesting technologies. Currently the most prevalent single-use harvesting technology, depth filters block at lower loading capacities with higher biomass concentrations. Higher contaminant concentrations also make depth filters more sensitive to batch variation, which can lead to 50% oversizing of filter area to compensate for fluctuating filtration capacities. That drives up costs and increases waste. Other new commercially available single-use cell-removal technologies such as centrifuges still lack capacity. For harvesting higher–cell-density cultures, a major technical breakthrough would be welcomed.

Figure 1: Filtration principle of dynamic body-feed filtration (DBF) with diatomaceous earth (DE) (LEFT) and conventional filtration (RIGHT)



Body Feed Filtration Successful for Plasma Fractionation

When we looked at other industries that have similar process needs — such as the plasma fractionation industry — we found that they often use body-feed filtration for clarification of solutions with high solids content. The first use of diatomaceous earth (DE) as a filter aid in fractionation of human plasma was reported over five decades ago (**3**). Since then, a number of manufacturing processes for production of intravenous immunoglobulin (IVIG), albumin, and clotting factors have been developed based on that technology (**4–6**). Fractionation uses the principles of selective precipitation by pH adjustment, ionic strength, addition of alcohol, and temperature shifts. Precipitates are removed by depth filtration often in combination with

Figure 2: Mean particle-size distribution of cell-free Chinese hamster ovary (CHO) culture supernatants from three different harvest days determined at different pH values; green curve = pH 7.0, red curve = pH 5.0; measurement performed using a Mastersizer 2000 system (Malvern Instruments)

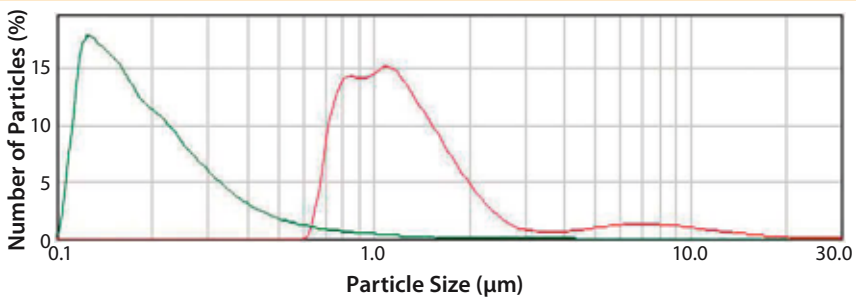


Photo 1: Scanning electron microscopy (SEM) shows the porous structure of Celpure 300 diatomaceous earth (magnitude 1,000 times).

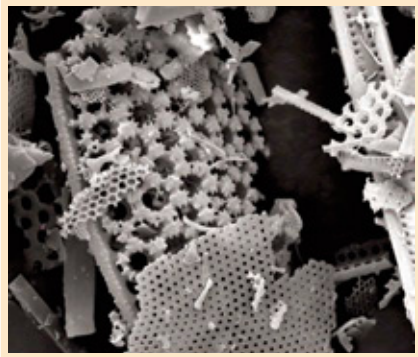


Figure 3: Turbidity measurement of the DBF filtrates at pH 7.0 and pH 5.0 for 10 different cell cultivations with an initial turbidity of 2,396–3,235 NTU

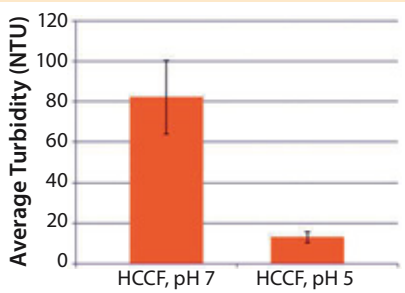
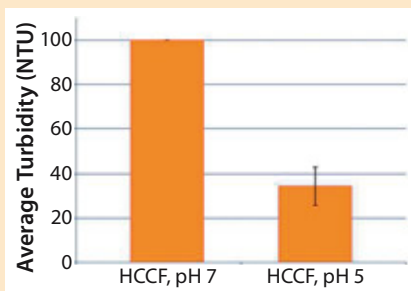


Figure 4: Mean residual DNA amount after DE filtration at pH 5.0 for 10 different cell culture supernatants, expressed in percentage of initial DNA concentration at pH 7.0, measured in the cell free supernatant; initial concentrations were 475–730 ppm (DNA quantitation with PicoGreen assay from Life Technologies).



diatomaceous earth, which is added as an aid to increase filter throughputs.

Recently, the principles of body-feed filtration have been tested for harvesting cell cultures, with promising results (7). Our objective was to evaluate the technology as a potential single-use alternative to centrifuges and depth filters. Here we describe the most interesting findings we obtained using a number of cell lines and culture media, which were kindly provided by different biotech companies. Together with Rentschler Biotechnologie GmbH, Sartorius Stedim Biotech tested the optimized conditions in a 600-L cell culture production process to evaluate the scalability of body-feed filtration technology.

How Body Feed Filtration Works

When the concentration of solids or colloids is too high in turbid biological process fluids needing clarification, the filter cake forming on the surface of a filter becomes impermeable and blocks the filter (Figure 1, RIGHT). Adding highly porous DE creates a more permeable filter cake, which prevents blockage (Figure 1, LEFT).

The minimum amount of DE to guarantee smooth filtration depends on the particle concentration. Laboratory-scale experiments with many different mammalian cell lines, culture media, and viabilities revealed a correlation between the wet cell weight (WCW) and the required amount of DE at constant pH (data not shown). For all tested cultures, the optimum DE concentration was in the range of 40–50% of WCW. In all cases, the filter-aid ratio could be reduced significantly to a range of 20–30% when pH was lowered to pH 5.

Low pH Precipitation Improves Clarification Results

Performance differences between acidified (pH 4.3–5.5) and neutral cell culture fluids for other cell-removal technologies such as microfiltration (4) and depth filtration (5) can be explained by precipitation of smaller particles at lower pH levels. The solubility of cell debris and negatively charged impurities such as DNA and host-cell proteins (6) decrease along with pH. Figure 2 shows the mean particle-size distribution for three different cell-free cell culture supernatants at neutral pH (green line) and after the pH of those cultures was lowered to pH 5 (red line). Lowering the pH leads to formation of bigger particles and makes the typical submicron particles (<1 µm) present at pH 7.0 completely disappear. Therefore, body-feed filtration would clearly benefit from lowered pH.

In addition to the significantly reduced filter-aid concentration, all tested acidified cell culture supernatants showed much clearer filtrates than their neutral counterparts (Figure 3). When we analyzed the particle-size distribution of neutral body-feed filtrates, we detected only small particles (<0.4 µm). Filtration with a 0.2-µm membrane barely reduced those turbidity values, indicating that very small particles are mainly responsible for the higher turbidity values of the neutral body-feed filtrations.

The absence of smaller particles in acidified culture supernatants probably is also the reason for higher filtration capacities at constant filter-aid concentrations. We assume that small particles deposit in the pores of DE particles, lowering the permeability and filtration capacity of the DE overall. By increasing the filter-aid concentration, we could increase its capacity for retaining small particles without losing the required cake permeability.

Significant reduction of filter-aid consumption at low pH is promising

Figure 5: Schematic representation of pilot-scale body-feed filtration

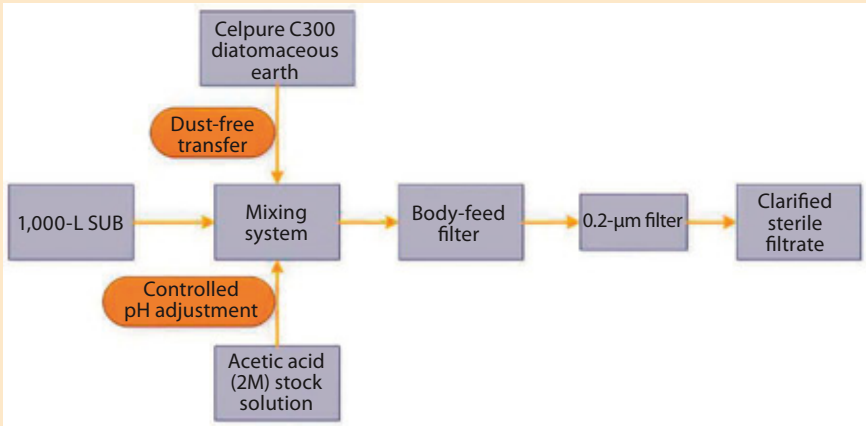
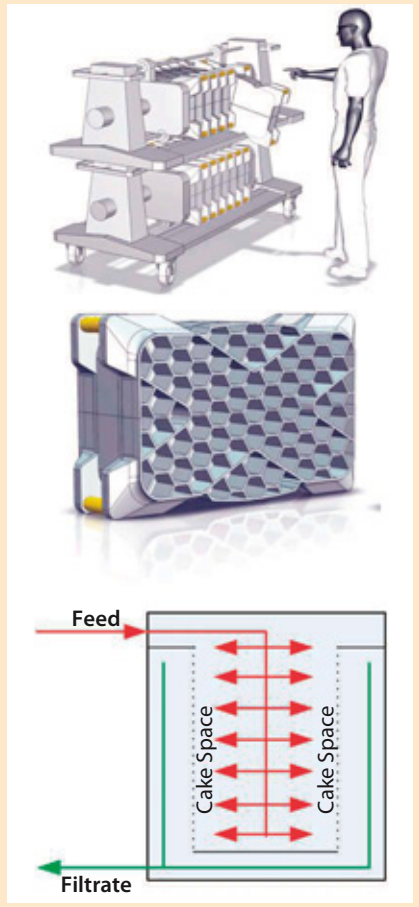


Figure 6: (TOP) 3-D illustration of commercial DBF process-scale module holder; (CENTER) a drawing of one single-use DBF module; (BOTTOM) flow path inside one module indicates in red the feed flow and in green the filtrate flow path; dotted lines indicate the filter plates.



from both economical and handling points of view. Another important economical factor to consider is the recovery rate at lower pH levels. Although most antibodies are stable at acidic conditions (**9**), in some cases low-pH precipitation of cell culture fluid will lower recovery rates, most likely due to coprecipitation of the antibody (**6**). However, some authors have described that lowering the pH value influenced overall antibody recovery positively by preventing enzymatic reduction of the product (**10, 11**). In all our laboratory-scale body-feed tests at low pH, we achieved a recovery rate >85% (data not shown).

Positive Impact on Residual DNA and Host-Cell Protein Load

With the low-pH precipitation and subsequent retention of contaminants by body-feed filtration, we accomplished a significant reduction of DNA in the primary recovery step from our cell culture runs. Figure 4 shows the mean residual DNA content of 10 different body-feed filtrates after low-pH precipitation at pH 5.0. We first measured the initial DNA content in the neutral, cell-free supernatant of each cell culture at pH 7.0. After a low-pH precipitation step followed by DE filtration, DNA content in the cell culture supernatants was reduced by 65%.

Pilot-Scale Test Results

At Rentschler Biotechnologie, we performed tests at pilot production scale to evaluate the applicability of DBF technology for manufacturing purposes (**12**). In total, 1,000 L of a high–cell-density (17.6×10^6 cells/mL, 95% viability) Chinese hamster ovary (CHO) cell fed-batch culture were available for the depth-filtration and body-feed filtration runs. In the largest single run, we subjected 600 L to body-feed filtration at pH 5.0. WCW on that day was 8%.

For the scale-up experiment, we installed seven process-scale modules with a total filtration area of 1.61 m^2 in a universal stainless steel holder. The filter cassettes consist of two polyethylene filter plates, which retain the DE and biomass (Figure 6).

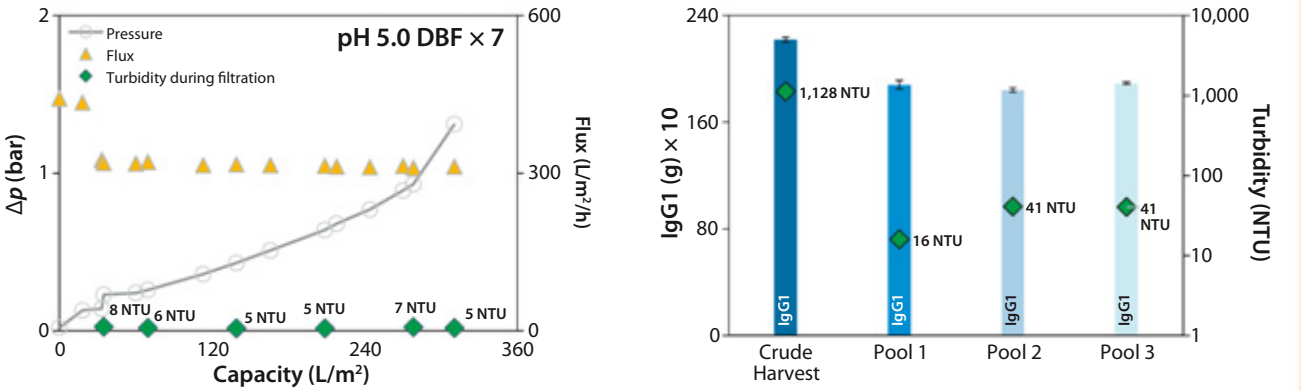
In total, we added 12 kg of Cellpure C300 DE to the 600-L bulk harvest. Prefilled DE bags were connected with a dust-free adapter to a mixing bag for fast and safe DE transfer. Just five minutes of gentle mixing was sufficient to dissolve that DE powder in the cell suspension. Before filtration, we adjusted the pH of the resulting mixture to a final level of 5.0 and gently mixed it for two hours at 140 rpm.

Pressure increased steadily during filtration (Figure 7, LEFT). We terminated filtration when the pressure reached 1.3 bar and the crude harvest had been filtered. During the entire filtration process, the system maintained a high and stable flux slightly above 300 L/m^2/h. Overall, a capacity of 311 L/m^2 was achieved. We monitored a very low turbidity of 5–8 NTU (Figure 7, LEFT) in the clarified harvest stream during filtration.

After neutralization, pool 3 (the final pool) exhibited a turbidity of 41 NTU (Figure 7, RIGHT), which was considerably higher than turbidity had been during filtration. Inadequate dosing of the neutralization buffer probably had resulted in local pH excursions and caused the turbidity increase in that final pool. In a small-scale parallel test, the turbidity increase was prevented through more gentle neutralization of the filtrate pool. To improve that neutralization step, an integrated ready-to-use process skid is under development that will enable controlled inline pH adjustment and prevent overshooting.

We found IgG1 recovery to be acceptably high at 85% (Figure 7, RIGHT). In the future, an optimized neutralization procedure and larger postfiltration flush should further improve MAb recovery. We monitored contaminants such as a host-cell protein and DNA throughout the process. In the final pool (after buffer flush and neutralization), those levels

Figure 7: Results of DE body-feed scale-up experiment at reduced pH (5.0) with seven filter modules; filtration performance (LEFT), pressure (bar), and flux (L/m²) as well as the course of turbidity during filtration; recovery of IgG1, blue columns (RIGHT) measured from crude harvest and harvest pool without buffer flush (pool 1), with buffer flush (pool 2), and after neutralization of harvest fluid (pool 3)



were reduced from 841 to 629 mg/mL and 13.8 to 5.0 µg/mL, respectively.

Concluding Remarks

Our aim was to demonstrate the universal applicability of a new single-use harvest method for mammalian cell culture, suitable even for high–cell-density cultures. Tests using crude harvests from different cell lines and culture conditions allowed us to determine the optimal concentration of DE as a filter aid in relation to WCW, which is an easily accessible process specific for cell removal and harvest processing. With respect to process economics, the 50% reduction of filter aid required with low-pH filtrations is promising.

Generally, the pilot-scale results confirmed our findings from DBF filtration trials at laboratory scale: Reducing pH to 5.0 after addition of DE to crude cell-culture supernatant gives the best performance in terms of filtration capacity, flux, and contaminant removal.

The applicable flux rate of DBF technology is very advantageous. A 600-L harvest was processed within only an hour of filtration using just seven modules. A module holder allows arrangement of a maximum 33 modules, so we estimate that a harvest volume of ~3,000 L could be filtered in the same time.

In conclusion, this method allows effective clarification of high–cell-density, crude cell culture harvests in a single-use set up at large scale. Economically and competitively, it can replace centrifugation, which is currently the method of choice for large-scale cell removal. Even very dense crude cell harvests could be clarified quickly at high flow rates. Moreover, this method has the additional benefit of efficiently reducing contaminants in a single step. Scalability — one of the most important requirements in bioprocessing — is easily attainable following a generally linear approach with very consistent process performance.

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Designing Single-Use Solutions for the Future

A Conversation with Christel Fenge

by Brian Caine and S. Anne Montgomery

Recently, BPI publisher Brian Caine and editor in chief Anne Montgomery had the opportunity to talk with Christel Fenge, Sartorius Stedim's VP of marketing for fermentation technologies, in the Göttingen, Germany, Sartorius facility. They began by talking about fermentation technology, a topic that led them to touch upon a number of key issues in the biopharmaceutical industry.

Left to right: Brian Caine (publisher of *BioProcess International*), Christel Fenge (vice president of marketing for fermentation technologies at Sartorius Stedim Biotech), and S. Anne Montgomery (editor in chief of *BioProcess International*).

Fermentation Technology

Caine: Because this special issue focuses on fermentation technology, let's begin by talking about some recent technological improvements and what impact these developments will have.

Fenge: The major change or "breakthrough" of the past decade is the increasing use of single-use technologies, which started in the early 2000s with the implementation of the Wave bioreactor (now known as the BIOSTAT RM [rocking motion] bioreactor). Given the increased attrition rates and development costs, better tools were needed to make clinical trial materials more efficiently. Subsequently, single-use stirred tank bioreactors were introduced, which led to a huge improvement in the way we develop biopharmaceuticals, particularly with the large monoclonal antibody (MAb) portfolios that many big pharmas have today.

Also, process analytical technologies (PAT) have been increasingly adopted by the biopharmaceutical industry, improving development times and providing a structured approach to fully understanding the production process. Other less regulated sectors such as "white biotechnology" have adopted PAT to a much wider extent to save costs, and I believe this will happen to our industry as well. For Sartorius Stedim Biotech (SSB), this means offering design of experiments (DoE) and multivariate data analysis software tools from Umetrics, enhanced with single-use sensors for pH, oxygen and online glucose and lactate measurement. Therefore, we also decided to acquire TAP Biosystems, and we can now supply ambr multi-parallel mini-bioreactors, highly automated bioreactor systems that can significantly increase the speed and efficiency of process development.

An Acquisitions Strategy: Fenge describes the acquisition of TAP and its multi-parallel bioreactor systems as being "very deliberate." She comments: "Our customers have a large number of development projects in their pipelines, and we wanted to be able to help them with process development. Clearly, highly automated, multi-parallel bioreactors fill a last remaining gap that hadn't been addressed in the past. We want to make process development faster, more effective and more cost-efficient. For me, this acquisition fits perfectly. It provides full and seamless scalability from 15 mL all the way up to 2,000 L in single-use stirred tank bioreactors: from cell line development and media screening at 15 mL scale, to

process development at 250-mL scale, process characterization and scale-up at 2 L, preclinical and clinical production at 50–1000 L, and finally, full production scale at 2,000 L. The ambr bioreactor has been very quickly adopted across a number of different product segments, which demonstrates a very real industry need; if they didn't need it, they wouldn't have adopted it."

Caine: Tell me about the development and maturation of your single-use bioreactors.

Fenge: We started with the Wave-style bioreactor — a simple pillow-shaped bag. Other companies were exploring the use of modified single-use mixers with noncentric mixing devices as stirred-tank bioreactors, and they launched the first single-use bioreactors (SUBs). We went one step further with the BIOSTAT STR, offering a true stirred-tank bioreactor based on the established design principles of glass and stainless steel vessels. For us, it was very important to create a bioreactor that provides the same scale-up characteristics and process performance that biotechnologists have been using for decades to make vaccines and drugs.

Seamless Scalablity: The major benefit for the customer, Fenge comments, is that it provides seamless scalability from process development (2–10 L) to commercial production and straightforward process transfer to existing large-scale production capacities. She adds that the BIOSTAT STR compares extremely well with classic stainless steel stirred-tank bioreactors, even in demanding processes such as cell culture on microcarriers or high cell density, concentrated fed-batch processes. The company is now in the process of launching the 2,000 L BIOSTAT STR for commercial production.

Ease of Use: "A key aspect for us during development was ease of use. Installing a 1,000-L or 2,000-L single-use bioreactor bag needs to be carefully considered; users are looking for easy, fast, and straightforward procedures that avoid complicated and risky bag manipulations. With the STR, you open the door, insert the bag (with a special lifting device for

the 2,000 L version), fix it at the bottom of the bag holder and at the motor drive, and off you go," she adds.

The 2,000-L STR bioreactor is based on the same design principles as the smaller 50-L to 1,000-L versions that have been on the market since late 2008. With the development of the smaller BIOSTAT STR family, members started the interaction with customers and what functionality is required for their various processes. "Collaboration partners in the vaccine industry and the MAb space discussed aspects such as impeller and sparger design, port types and sizes, connectivity, exhaust lines and biosafety concepts with us, and what capabilities and technical solutions they are looking for. Our process scientists frequently liaise with customers to gain a deep insight into their processes and needs, which is extremely useful," said Fenge.

Montgomery: What are your thoughts about the scalability of single-use bags: in practical terms, is 2,000 L the uppermost limit?

Fenge: Yes, we believe so for various reasons. High titers (5–10 g/L) and the trend toward smaller indications, personalized medicine, and regional production make 2,000 L a very attractive option. Another trend is the move toward concentrated fed-batch, a type of continuous culture in which the product remains in the bioreactor, and that enables very high product titers. Typically, companies want to benefit from single-use and smaller bioreactors to make their processes more manageable, make capacity adaptation more flexible, and derisk process transfers.

Film Strategy

Caine: In connection with the 2,000-L BIOSTAT STR version, I understand you developed a new film, especially for this large-scale single-use bioreactor.

Fenge: During the past two years, we have been working on our "film strategy." This is a materials science project for future single-use manufacturing. As the use of single-use bioreactors in commercial production increases, our customers are looking for new levels of assurance of supply and business continuity when it comes to components and raw materials. They are concerned about change control; namely, how we as a vendor control and manage changes in our production and supply chain. Companies are now "outsourcing" activities that, in the past, they used to control themselves. And given the single-use nature of today's bioreactors, manufacturing them is a recurring process and not a one-off event covered by installation and operational qualification (IQ/OQ) as it was in the past when commissioning stainless steel production capacity.

As such, we are committed to understanding and controlling the whole production process and the quality requirements that go with it. One aspect is raw material traceability, right down to the polymer resins, to where the polymers are manufactured, establishing traceability and control over the entire process from the resin and the additives to the final bag. We want to ensure, for the long-term, that the film material we use in our bags is of consistent quality. We achieve this by establishing specifications and controls for the polymer resins and additives that are used to manufacture the new film and by establishing process understanding and control of the film extrusion, bag assembly, and gamma irradiation processes.

From a few milliliters up to 2,000 L, Sartorius Stedim Biotech covers the complete product range of bioreactors for upstream processing.

Montgomery: Can you give us some specific characteristics about your Flexsafe bags and the new polyethylene film?

Fenge: The new Flexsafe STR bags were an important component in the development of the 2,000-L bioreactor and its application in commercial production. It was important for SSB to ensure the consistent supply of a dependable polymer film that would provide the robustness and assurance of supply that people look for when using the product in full-scale manufacturing.

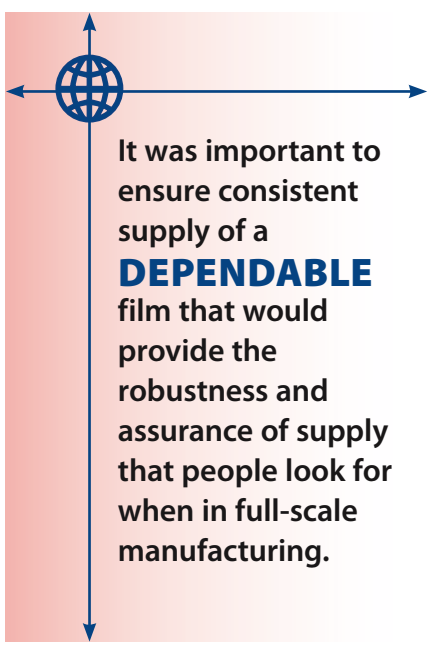
One key driver was the sheer size of the 2,000-L bag, which forced the company to look into a new film and our desire to stick to the same geometrical design principles of the existing STR product range. We also knew that our customers had experienced poor cell growth with certain (other) bags, so we — and our industry partners — focused on that when we developed the new polyethylene (PE) film. It turned out that certain antioxidants degrade to substances that are toxic to cells, even at very low levels, so we optimized the antioxidant package to ensure that we can consistently supply single-use bioreactor bags that deliver excellent cell growth. Another key aspect is robustness; bags need to reliably work in day-to-day operations.

Our new PE film is the thickest in the industry and the most robust. We try to balance its stiffness or rigidity with flexibility. A bag has to be flexible, but also rigid enough to work in a three-dimensional large-scale bioreactor, mixer, or shipping bag. It must be resistant to both hydrostatic pressure and liquid movement, which is why we spent almost two years developing the new PE film. We wanted to put the key elements of traceability, control, assurance of supply, and robustness firmly in place. And right from the beginning, we wanted to have a PE film that works for a broad range of different bioprocessing applications, including stirred bioreactors, Wave-type bags, large mixing and shipping bags, storage and freeze-and-thaw bags, to reduce the validation effort of our customers.

Caine: How do you address customer concerns about extractables and leachables?

Fenge: We typically provide our customers with an E&L guide. Under a confidentiality agreement, they get access to model liquid (water and ethanol) data. An E&L guide is available for each bioprocessing bag type and covers the E&L analysis for the complete bag, including the film, tubing, connectors, and/or, in the case of the Flexsafe STR, the agitation and sparger system components. Customers can then decide what data to submit to the regulatory agencies, using either our model data or data for their specific process fluids. SSB can also perform process-specific E&L studies with customer liquids. With the new film, we've taken an even more rigorous approach; as a supplier, you cannot provide reliable E&L data if you don't have full control of your polymer resin, additive package and film and bag manufacturing process. Only by doing so can you assess whether the E&L profile for one batch is representative of future batches. With full control, we can take the E&L discussion to a new level.

Materials Composition and Sourcing Issues: Many companies are still rather naïve regarding how the films are made, what the sourcing issues are, and how variations in composition might affect their products. Fenge comments that "manufacturing your own film helps to provide greater customer assurance. If a company is going commercial, it will need to supply patients for years. We're not talking about a clinical trial; we're talking about guaranteeing the supply for full-scale, commercial production, for the long-term. Companies should talk to us about the materials science involved, about the film manufacturing process, and about the controls we have in place. As single-use technologies mature and move into commercial production, the industry is demanding assurance of supply. They want to ensure that their vendors can provide them with single-use bags that show consistent quality and reliable performance over and over again," says Fenge, "Putting raw material specifications in place and



establishing operating ranges for the extrusion process reflects the cGMPs that our customers use in their own manufacturing.

"We feel it is extremely important that we, as suppliers of single-use components and solutions, fully understand what they will be used for, as well as the significant value of the pharmaceutical intermediates and products being used in them. Understanding the product profile is important: What is the intended use, what are people doing with it, what do they expect? As such, we communicate with our end users. Some companies even come to our production site to talk about their drug manufacturing processes and products; we try to establish a connection with the end users," adds Fenge.

Standardization

Montgomery: Do you get many questions about the compatibility of your materials with those of other single-use manufacturers?

Fenge: Yes, absolutely. We currently incorporate components from various vendors. Our customers want interchangeable systems that can use both our bags and our competitors' bags, so it's all about standardization. And as much as we try to accommodate the needs of our customers, it makes the supply chain very challenging and involves a lot of manual work and risk assessments, which adds to the cost. In the past, this was not a major concern because most single-use bioreactors and bags were used in clinical manufacturing. And because it is a relatively young technology, there is a lot of innovation, and people want to test different solutions or simply come up with their own designs. But achieving the level of assurance and robustness of supply that's needed for commercial production when faced with all these different components and modifications — that's a different matter. Customers should consider this aspect very seriously as their use of single-use solutions increases, which is why we talk to our customers about standardization. It makes the supply chain easier and more robust, not just for us, but also for our customers. It

helps to achieve price security, and most of all, customers can rely on tested process solutions whereas bespoke solutions might include unidentified process risks, especially for more complex single-use applications or whole unit operations such as bioreactors.

Assessing Risks in Single-Sourcing: For an end user, from a validation standpoint, it does mean releasing control, particularly if they're using Sartorius as a single-source solution provider. "It might require a leap of faith," admits Fenge, "and this is something that definitely needs ongoing work. But single sourcing is not new to the industry; it's common practice for critical components such as microcarriers, chromatography gels, and virus filters. It's really about risk assessment, balancing risks and benefits, and ultimately, risk management through (for example) the use of safety stocks, close collaborations, and quality agreements between suppliers and end users. As a supplier, we are committed to building trust in our materials strategy and quality assurance. We've not yet reached the end of the innovation stage in single-use and/or the level of maturity that is necessary to standardize, so there's still quite a lot of work to be done."

Long-term film supply and disaster preparedness is another critical consideration. But Fenge feels confident about addressing business continuity through a mix of establishing back-up raw materials for the polymer resins, back-up film extrusion and bag manufacturing capabilities, and safety stocks of resins and film rolls. "There are two elements to the new film strategy," she says. "One element is controlling how the film is manufactured, which ensures batch-to-batch consistency; and the other is the business continuity element, which is based on long-term agreements with our extrusion and resin partners, safety stocks, and back-up capabilities."

Media Advances

Caine: You acquired Lonza's cell culture media business at the end of 2012. Tell us about the role that's going to play and how that helps you to become a total solution provider.

Fenge: One aspect that we couldn't supply in the past was cell culture media. But given our desire to provide a "total solution," customers can now buy liquid media from us in our bags, made with our filters. Or alternatively, they can use powder media, making their media at the point of use using our Flexact MP unit, filters, and bags. We are also developing further applications in this area based on the acquisition of the ambr multi-parallel bioreactors. Our goal is to bring added value to our customers, and so far the introduction of a media offering has gone extremely well, especially with our key accounts. Very often, they will use their own cell culture medium but might be looking for a second supplier, which provides an opportunity to discuss buying it from us. Of course, we are not well known as a cell culture media company. But Lonza has years of media (powder and liquid) development and manufacturing experience. Working together, our combined market reach, expertise, and capabilities enable us to support our customers even better.

Long-Term Growth Strategy: The media offering is part of the company's 2020 strategy, a key component for future growth. And Fenge acknowledges that having the long-term strategy to be a total solution provider means that Sartorius has been quite aggressive in the short-term to put all the pieces of that strategic plan — the vision — together.

Expanding Capabilities

Looking ahead, Fenge expects the company's single-use capabilities to grow and expand into other areas, such as the production of ADCs, cell

SSB's ambr15 system enables rapid evaluation of 24 or 48 multiple bioreactor cultures at microscale.

therapies, biosimilars, and so on. "We're keeping a close eye on those applications, and in fact, we're already very active in biosimilars and the ADC field. We've recently engaged a Regenerative Medicine Taskforce to work specifically with that market segment and position our existing products there more strategically than we've done in the past. We also envision selected developments or adaptations. Regarding cell therapy, for example, I don't think you can really do cell therapy without using closed, single-use systems. We are also working with Lonza and other collaboration partners to demonstrate good stem cell propagation. We strongly believe in the suspension route for scale-up, which doesn't necessarily mean that you have to grow the cells in single cell suspension. But ultimately, we believe very strongly in stirred tanks. You can control them. It will be interesting to see what develops and becomes standard procedure in the years ahead."

Bag Testing and Packaging

Montgomery: How does your new bag tester ensure process safety, and how does your new packaging concept fit into that picture?

Fenge: Beyond talking about materials science, consistency, control, and business continuity, we thought about how else Sartorius Stedim Biotech could help customers to derisk their use of single-use systems. All things considered, it's a bag. But special considerations are necessary for the transport, storage, unpacking, and installation of that bag, so we have developed a device that allows operators to pressure test their bioreactor bag assembly, postinstallation but preuse; it's very similar to the approach that many users take for their steel tanks. So far, we have qualified the bag tester for our CultiBag STR bags, from 50 to 1,000 L, and qualification for the new Flexsafe STR bag range (up to 2,000 L) is in progress. In addition, we are shipping our STR bags overseas to destinations such as North and Latin America, Australia, China, Korea, and India, to places where the roads are not always in the best shape. Our new packaging concept helps to ensure safe transport and mitigates any risks of damage during transportation. It has passed the very stringent ASTM shipping validation conditions.

Caine: What you think the future is going to hold for single-use technologies?

Fenge: I believe that we will see an increased level of automation and a move toward automated single-use facilities. This will reduce manual handling. Single-use sensor technologies are also improving, and so I think we will see more industrialized processing, comparable with the routine production of chemical APIs. We will see a push toward the so-called ballroom concept, which will help to address cost pressures. With robust data on the safety of closed single-use systems, we will also be able to help our customers to present the ballroom facility concept to the regulatory agencies.

Enabling Global Expansion

Finally, Fenge describes the use of single-use technologies as an enabler that will provide the opportunity to expand drug development and manufacturing applications around the world for many types of disease treatments. "As emerging economies grow stronger, their population wants access to the kinds of medications that we are already enjoying today in our developed world. So absolutely, single-use is definitely here to stay," she concludes.

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Consistently Superior Cell Growth

Achieved with New Polyethylene Film Formulation

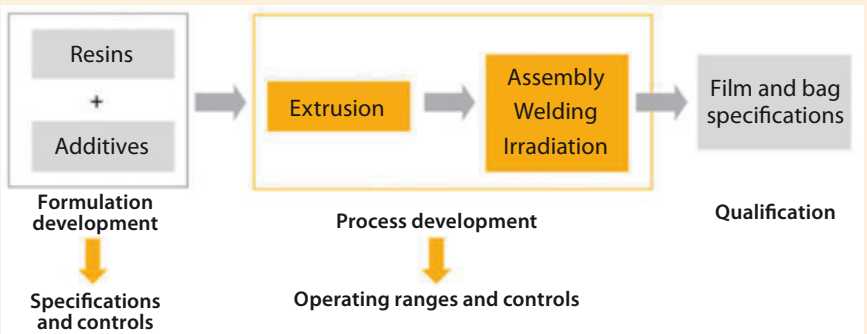
by Christel Fenge, Elke Jurkiewicz, Ute Husemann, Thorsten Adams, Lucy Delaunay, Gerhard Greller, and Magali Barbaroux

During the past decade, single-use bioprocessing bags and bioreactors have gained a significant foothold in the biopharmaceutical industry because they offer a number of advantages over traditional stainless steel equipment, especially for clinical production, multiproduct facilities, and emerging economies. At the same time, some companies are concerned that plastic materials might release potentially toxic substances that could affect cell growth and product titers (**1**). In a worst-case scenario, they could even compromise drug safety when a company uses disposable bags for intermediate or drug substance storage.

Despite the broad acceptance of single-use bags in clinical and commercial biomanufacturing, some researchers have reported recently on inconsistent and poor cell growth of some production cell lines in various single-use bags (**2–4**). Hammond et al. identified a degradation product derived from a commonly used antioxidant that can be present in some commercially available single-use bags at levels that negatively affect cell growth (**5, 6**). However, such antioxidants are necessary to keep single-use bags stable: They protect the polymer film during gamma irradiation and protect it from oxidative degradation during extrusion and storage. Hence, it is extremely important to optimize and closely control the concentration of such antioxidants to prevent batch variability.

Other common additives are so called "slip agents," typically oleamide, erucamide, and stearamide. Such agents act as lubricants and prevent stickiness of polymer film layers, which in turn facilitates bag fabrication and in-process use. Ideally, slip agents should be omitted to further reduce the risk of leaching substances that could potentially affect cell growth.

Figure 1: Polyethylene film development and manufacturing strategy



Innovative Strategy

When Sartorius Stedim Biotech began development of its innovative S80 polyethylene (PE) film for the new Flexsafe range of bioprocess bags, the company's vision was to establish full control and traceability of resins and additives, to fully control the manufacturing process (including film extrusion), and to guarantee industry-leading security of supply to ensure business continuity for the biopharmaceutical industry (Figure 1). The aim was to move away from the current industry practice of using resins or films based on trade names, which in essence provides no true control of polymer and additive formulations. This new film ideally should work for all single-use bag applications, both in upstream and downstream. So it should meet the various physical, chemical, and biological requirements of culture media and chromatography buffer mixing and storage, stirred-tank and rocking-motion bioreactors, intermediate and bulk drug product storage, shipping, and freeze–thaw applications (as described in the "Target Product Profile" box).

Target Product Profile of New S80 Multilayer Polyethylene Film

Reproducible cell growth (comparable to that in borosilicate glass)

Traceable and controlled polymer and additive formulation

Robust and suitable for all single-use bioprocessing bag applications in upstream, downstream, and final filling:

- rocking-motion bioreactor bags
- stirred-tank bioreactor bags
- two- and three-dimensional (2-D and 3-D) storage bags
- mixing bags
- shipping bags
- freeze and thaw bags

Standardized Cell-Growth Assay

Protein-free, chemically defined cell culture medium incubated for three days at 37 °C in freshly γ-irradiated sample bags at a volume/surface ratio of 3 cm^2/mL

Control: medium incubated three days at 37 °C in Duran glass bottle

Method: rCHO DG44 cells grown in six-well plates using the above treated medium

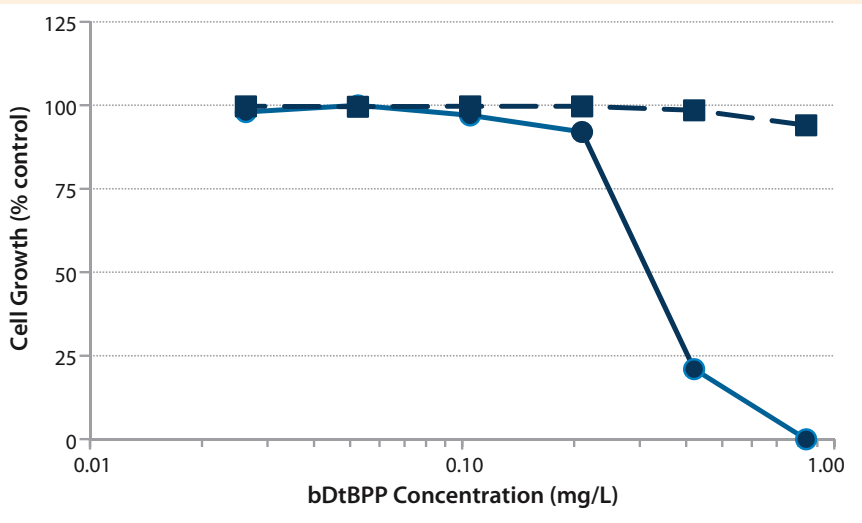
To achieve those goals, Sartorius Stedim entered into a strategic partnership with Südpack, Europe's leading film-extrusion company. Furthermore, we established a strong collaboration with leading polymer and additive manufacturers to access and control the formulation of our polymer and additive package. That is crucial to a strong assurance-of-supply concept.

Development of New Film

Standardized Cell-Growth Assay: During development, 25 different polyethylene multilayer film formulations (based on widely used polymers and European Pharmacopoeia listed antioxidants) were created and evaluated against the defined target product profile (TPP) of the new film. To identify potentially harmful leachables, we established a standardized cell culture assay to examine gamma-irradiated sample bags, as described in the "Standardized Cell Culture Assay" box (**3**, **4**). We understood early on that the typically conducted USP <87> cytotoxicity testing would not be predictive of cell growth behavior.

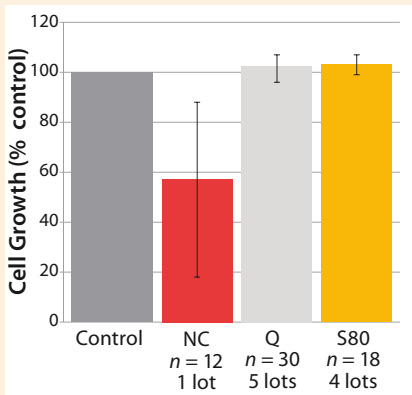
No Slip Agents and Optimized Antioxidant Package: Instead of slip agents, we applied nontoxic mechanical antiblocking based on silicon dioxide, which creates a slightly rough surface to counteract stickiness. We optimized the antioxidant package and minimized the concentration of a pharmacopeia-listed, trisarylphosphite-based processing stabilizer (sold under a number of trade names). That component is known to degrade into a reported growth inhibitory substance (**5**): bis(2,4-di-tert-butylphenyl) phosphate (bDtBPP).

Figure 2: Cytotoxicity of bDtBPP; CHO-DG44 viability (dotted line) and cell growth (solid line) as a function of bDtBPP concentration (**7**)



Most important, we established the dose dependency of cell growth and viability for a model recombinant Chinese hamster ovary (CHO) cell line as a function of bDtBPP concentration (Figure 2) to ensure that our cell growth assay would identify nonsuitable formulations, which were evaluated during development of the polyethylene film. Figure 3 illustrates results from some selected film formulations. We found the "S80" formulation to be the most suitable, showing no impact on cell growth in our standardized assay or with other production cell lines (**4**, film 6).

Figure 3: Cell-growth behavior in some selected film formulations; a number of film lots and sample bags were evaluated. The "error bar" shows minimum and maximum cell growth observed.

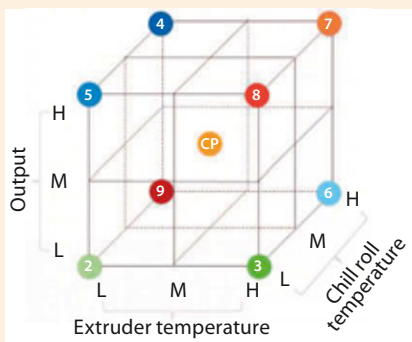


Extrusion Process Operating Ranges Established

Next, we assessed the impact of extrusion process parameters on cell growth behavior within a defined window. We also performed an expert risk assessment of those parameters and their likely effects on film quality attributes.

The main influencing factor during film extrusion is the amount of heat transferred to process polymer materials. This heat transfer is directly proportional to the oxidation of additives and the quantity of degradation products that are generated to protect the polymer chains. This heat transfer is linked to three critical extrusion process parameters: the melting temperature of resins (extrusion temperature), the cooling temperature of the film (chill roll temperature), and the quantity of material extruded per hour (output).

Figure 4: Graphical representation of full factorial experimental design to establish operating ranges of critical extrusion process parameters (extruder temperature, chill roll temperature, and output); L, M, and H represent low, middle, and high settings. Center-point (CP) settings were performed in triplicate. (**7**)



We used the best film formulation determined in our initial studies and conducted a full multifactorial experimental design (Figure 4). We examined cell growth using our established cell-growth testing approach, demonstrating that the evaluated critical process parameter (CPP) ranges provide consistent cell growth comparable to the control sample results (Figure 5).

No Aging Effects Detected

Because degradation of antioxidants continues during storage of film rolls, after γ-irradiation and dry storage of bags, we investigated the cell-growth performance of our selected film formulation in an accelerated aging study and compared the results with those of a competitor's bag. Most notably, our S80 film formulation ensures excellent growth behavior right after γ-irradiation until the end of the aging study — representing 36 months (Figure 6) — whereas the competitor's film results showed an initially poor growth behavior that did recover after six months of dry storage. That suggests further degradation of bDtBPP into nontoxic compounds. So our data support bag reliability from day one until the end of shelf life — batch-to-batch consistency, in other words — whereas other vendor bags may show deviations depending on storage time.

Figure 5: Influence of critical film extrusion process parameters on cell growth examined using the established cell-growth testing approach; error bars represent ±SEM (standard error of mean). The numbers correlate to different experimental conditions of the factorial design; 1a, 1b, and 1c represent the center-point setting. (**7**)

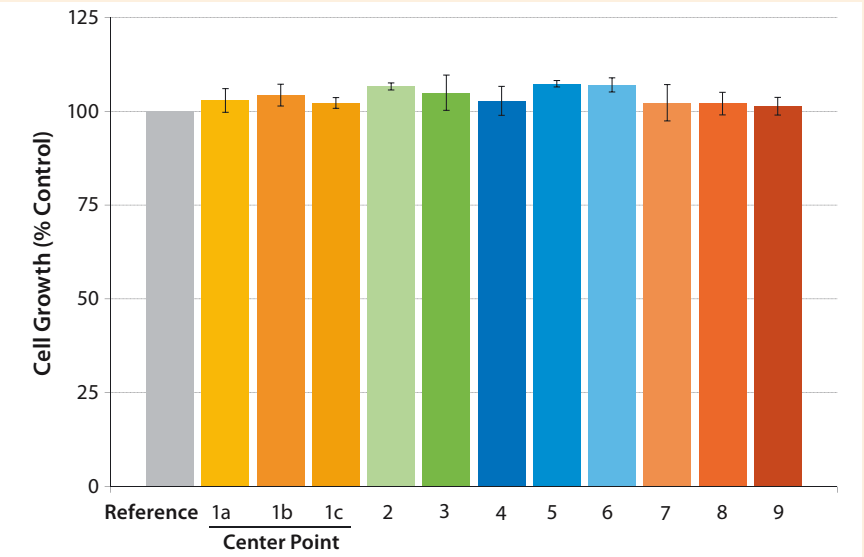
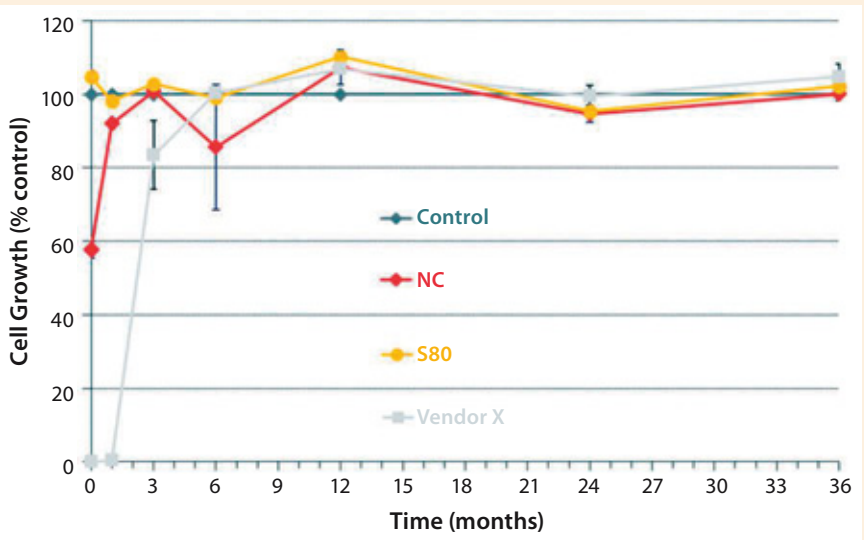


Figure 6: Cell-growth behavior of γ-irradiated bags stored under accelerated aging conditions (36-month representing 337 days at 40 °C); optimized film formulation (S80) showed excellent growth right after γ irradiation until the end of the study (representing 36 months). The negative control (NC) and a bag from another vendor showed initial poor cell growth that recovered after about six months. (**7**)



No Interaction with Culture Media

This new polyethylene S80 film should work across the full range of bioprocessing applications. Therefore, we were especially interested in understanding cell-growth performance in cold-storage applications with protein-free, chemically defined cell culture media. We considered that to be a worst-case scenario given that serum, hydrolysates, and protein additives can mask detrimental effects of toxic leachates. Gamma-irradiated test bags and borosilicate bottles (control) were filled with chemically defined medium and stored at 2–8 °C for up to six months. No negative effects on cell growth were detected using our cell growth assay (Figure 7) during the storage period.

Quality by Design Approach to Establish Film Specification

Using a quality-by-design (QbD) approach, we established a film specification that ensures excellent and reproducible cell growth, proven by a broad panel of different production cell lines and cell culture media. Together with our strategic collaboration partners in the polymer and film industry, Sartorius Stedim Biotech established full control of the new, state-of-the-art, polyethylene multilayer film formulation as well as traceability and control of polymers and additives used in it. We applied a multifactorial experimental-design approach to determine operating ranges of the film extrusion. And we established operating ranges for film-welding parameters and γ-irradiation of Flexsafe bags (data not shown). Based on this unprecedented material

and biological science and process control approach, we can ensure excellent and reproducible growth behavior for the most sensitive cell lines with Sartorius Stedim Biotech's Flexsafe bags. This has been confirmed by a recently published interlaboratory test (**4**), in which a number of bioprocessing bags from different vendors were evaluated using a panel of different production cell lines and media. Other authors have demonstrated the suitability of Flexsafe bags for mesenchymal stem cells (**8**).

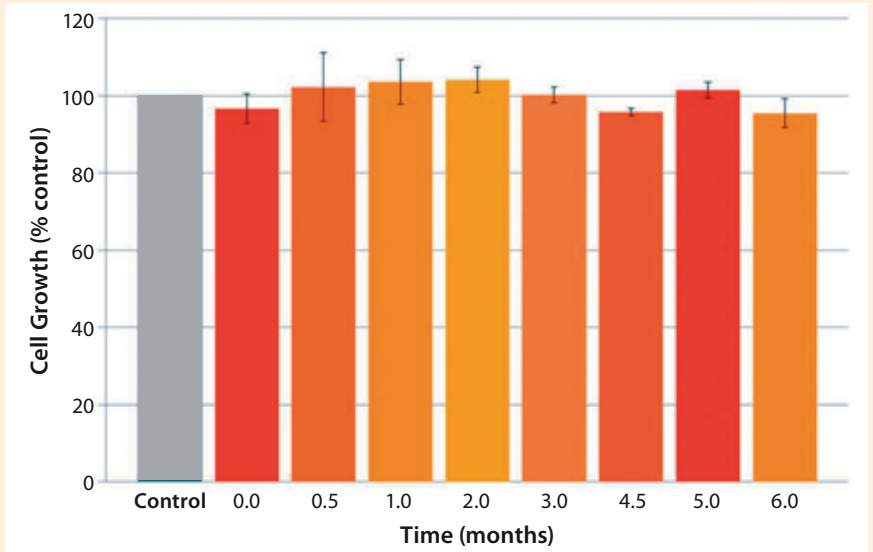
Controlling the complete manufacturing process — from resin to final bag — ensures consistent and reproducible quality for the new generation of Flexsafe bioprocessing bags made with the innovative S80 film. This has been proven by lot-to-lot consistency in cell-growth performance of this new multilayer polyethylene film. Understanding and controlling the film formulation and manufacturing process are key prerequisites to establish meaningful and reliable extractable and leachable data as needed for toxicological assessment of intermediate and bulk drug substance storage and stability studies.

All that combined with a robust assurance of supply concept guaranteeing uninterrupted supply of bioprocessing bags (**9**) is absolutely crucial to pave the way toward fully single-use bioprocessing facilities of the future. It ensures that a reproducible quality of bioprocessing bags can be supplied throughout the entire life cycle of a biologic drug — from clinical development, when single-use bioreactors and bags might be used for the first time to make or store investigational medicinal products, to many years after market launch of the final drug product.

Conclusion

The material science and process understanding applied during development of the new S80 film — enabled by strategic partnerships in the polymer industry — were cornerstones to delivering the new Flexsafe range of bioprocessing bags.

Figure 7: Cold storage (2–8 °C) of chemically defined cell culture medium in S80 polyethylene film bags; excellent cell growth (comparable to control) was detected over the entire storage period (**7**).



This unprecendented traceability and control aproach answers key needs of the biopharmaceutical industry for single-use factories of the future. Sartorius Stedim Biotech's new S80 polyethylene film sets the standard with respect to cell growth, robustness, assurance of supply, and batch consistency that are unprecedented in the industry.

Acknowledgments

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Robust and Convenient Single-Use Processing

The Superior Strength and Flexibility of Flexsafe Bags

by Elisabeth Vachette, Christel Fenge, Jean-Marc Cappia, Lucie Delaunay, Gerhard Greller, and Magali Barbaroux

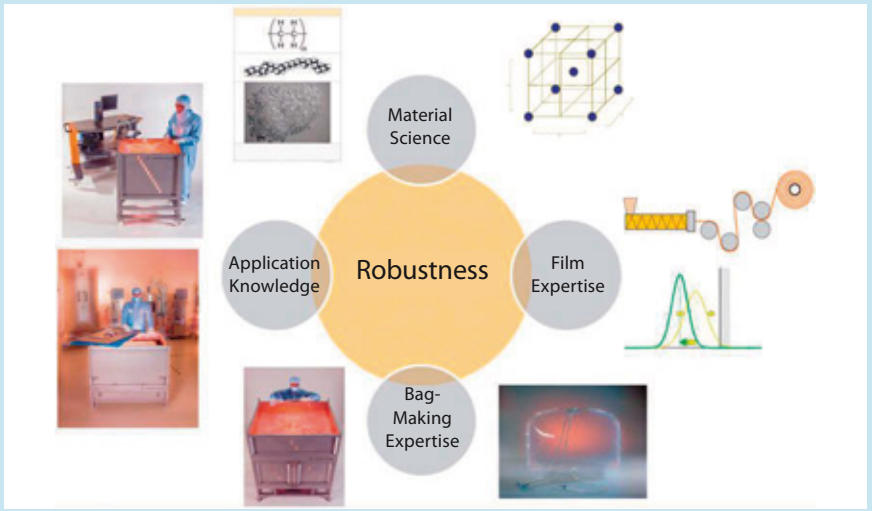
With the increased use of disposable bioprocessing bags in all critical process steps of the biopharmaceutical drug production, there is a growing requirement for high-quality, robust, and easy-to-handle bioprocessing bags. The new generation of films and bags must combine multiple mechanical, physical, and chemical properties to make these products suitable and scalable for all processing steps in upstream, downstream, and final filling operations, including cell culture in rocking motion and/or stirred-tank, single-use bioreactors as well as storage, mixing, shipping, and freezing.

In partnership with our resin and film suppliers, Sartorius Stedim Biotech's polymer and film experts have selected the best raw materials, optimized the design space of the extrusion process, and defined the critical welding parameters following the principles of quality by design (QbD). With this approach, we have developed a new polyethylene film and new bags that combine strength and flexibility, the two prerequisites that will provide for robust and convenient single-use bioprocessing.

The robustness of our new S80 polyethylene film structure (used in manufacturing new Flexsafe bags) has been demonstrated by means of standard flex-durability, tensile strength, elongation, and energy at break testing. Furthermore, we applied a water-burst test developed in house using model bags and worst-case application testing of two-dimensional (2-D) rocking-motion cell culture bags and three-dimensional (3-D) stirred bioreactor bags to confirm the robustness of new Flexsafe bags in different applications. Notably, our new Flexsafe 3-D shipping bags passed the most stringent ASTM D4169 test for air and truck shipment.

The thickness, strength, and flexibility of S80 film improve its mechanical robustness and make it suitable for all bioprocessing applications. Its strength significantly reduces the risk of accidental damage to bags due to inappropriate handling. And the flexibility enables convenient installation and self-deployment of each bag in its container.

Figure 1: Film and bag development based on a quality by design (QbD) approach, with expertise in material science, application know-how, film, weld, and bag making



Industry Demands

The need for product quality, robustness, and integrity has been ranked in a number of surveys as the primary bioindustry requirement (1). Because single-use bags offer multiple technical and economic benefits, they are broadly adopted throughout the biopharmaceutical drug industry to help companies achieve rapid and flexible development and commercial production of monoclonal antibodies (MAbs), other recombinant proteins, and vaccines. The biotechnology industry is now expanding its implementation of single-use bags into all bioprocess steps for applications such as cell culture, storage, shipping, mixing, freeze and thaw, and final filling (2).

With that growing implementation of single-use bags, requirements are

increasing for outstanding product quality, assurance of supply, change control, robustness, ease of use, and scalability. These are important throughout all applications of single-use bioprocessing, from cell culture to shipping of bulk drug substances and final filling of drug products.

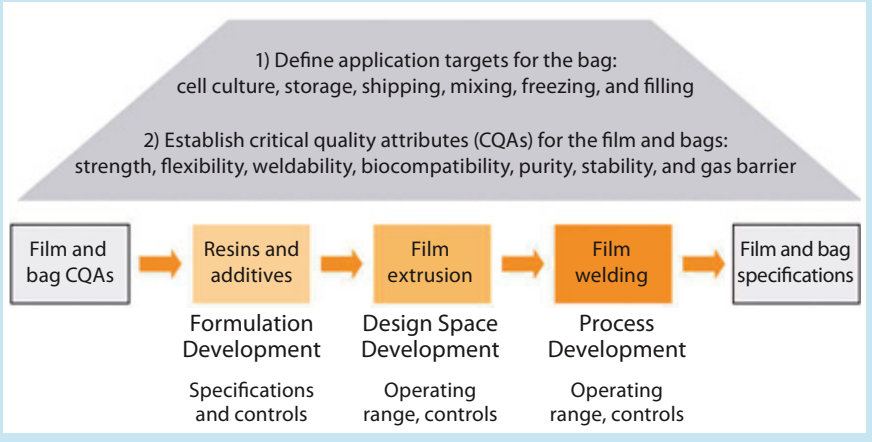
Partnerships with Resin and Film Suppliers

Developing innovative film and bioprocessing bags that meet all those emerging demands requires different disciplines and skills and expertise. So the new S80 multilayer polyethylene (PE) film from Sartorius Stedim Biotech has been specified and designed in partnership with Südpack, a leading film manufacturer, and in close collaboration with polymer and additive suppliers. This collaboration combines material science, application know-how, film-extrusion knowledge, and welding and bag-making expertise.

The resins in each layer of the S80 film and their associated extrusion parameters were selected after extensive prototyping in which various polymers and film compositions were evaluated to achieve the best mechanical properties. Our main objective was to obtain the highest film robustness: an optimal combination of strength and flexibility with the most reliable and robust welds. That in turn enabled us to design completely new bioprocessing Flexsafe bags suitable and scalable for all bioprocessing steps in upstream, downstream, and final filling. Flexsafe bags meet user-requirement specifications (URS) of both rocking motion and stirred-tank single-use bioreactor applications as well as medium, buffer, and intermediate storage; shipping, mixing, and freezing; and aseptic filling of bulk drug products.

Development of this innovative Flexsafe bioprocessing bag range followed a QbD approach to achieve reliable and robust bioprocessing bags suitable for all applications. This approach included selection of appropriate resins and additives and subsequent establishment of specifications and controls, definition of the film-extrusion process design space, and validation and control of the welding and overall bag-making process.

Figure 2: QbD approach to ensuring consistent robustness of Flexsafe bioprocessing bags



Quality by Design Approach

As described in ICH guidelines Q8, Q9, and Q10 and applied to new product development, a QbD approach ensures that developed products consistently meet intended performance criteria (**3–6**). Well-established and open supplier relationships and knowledge sharing are critical for selecting appropriate raw materials, identifying relevant specifications, and establishing necessary controls to achieve consistent robustness of the final single-use bag.

Our QbD approach to achieving consistent robustness for Flexsafe bags started with an in-depth understanding of the intended application and definition of the URS (Figure 2). Today, nearly all unit operations in biopharmaceutical drug development and production can be performed using single-use solutions. However, the required mechanical and physical properties can vary greatly by application. For example, a bag used in rocking-motion cell culture or in liquid shipping applications requires a very flexible film that resists material fatigue. A bag used in large-scale mixing applications or in a stirred-tank bioreactor design must absorb the significant hydrostatic pressure of 2,000–3,000 L of fluid. In such an application, film and weld strengths are more critical.

Further to bag robustness, routine use in production facilities calls for easy, convenient, and straightforward installation and robust handling tolerance. Here, film flexibility and easy bag deployment during installation and filling are the main targets.

The critical quality attributes (CQAs) we identified for providing robustness and ease of use in all bioprocessing applications were strength, flexibility, and weldability of polymer film. Strength significantly reduces the risk of accidental bag damage from inappropriate handling. Flexibility enables easy handling and installation and bag self-deployment in containers.

Material Science and Film Expertise

The superior robustness of single-use Flexsafe bags resulted from combining different competencies and capabilities to develop a completely new film that covers all aspects of their creation: from the choice of raw materials to final manufacturing of bioprocessing bags. Development of S80 PE film started with translating target application needs into URS, then into functional specifications for the bag, the film, and the resin and additive package.

Deep understanding of the underlying material science and expertise in polymers is required to select the right plastic resin materials and additives that will provide the desired quality attributes. As a first step, based on theoretical considerations grounded in material

Figure 3: Tensile strength data expressed in Newtons (N) for S80 and other PE films

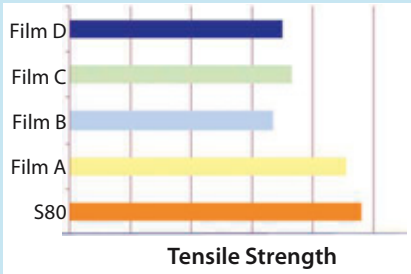


Figure 4: Elongation at break data of S80 compared with other PE films; elongation at break is shown in % of initial length of a film sample at the start of the experiment.

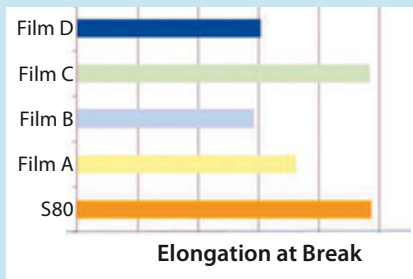
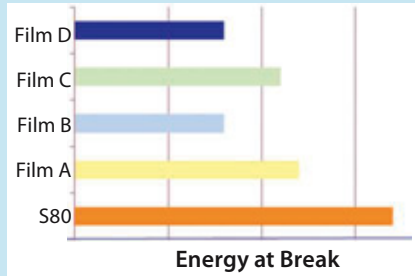


Figure 5: Energy-at-break data for S80 compared with other PE films



expertise, robustness is designed into the polymer by those who know the plastic materials and how they behave when brought together in a multilayer film. Our polymer experts tested and selected the plastic materials that confer the best film properties: strength, flexibility, weldability, biocompatibility, purity, gas-barrier properties, and relationship with drug stability. This material selection — the number and arrangement of plastic layers and their thickness together with the extrusion approach of the multilayer film — ultimately determined the properties and overall performance of the final multilayer film structure.

In-depth knowledge of film behavior helped us choose the right plastic material mix, considering that some of those necessary properties could be antagonistic. For example, a strong and stiff film is highly resistant to impact and puncture but shows less flexibility and resistance to fatigue. A too-flexible film will not withstand hydrostatic liquid pressure in large-scale bags. Our film experts designed several film formulations and combinations and thoroughly tested them against predefined CQAs. For the S80 film, the main CQAs for characterizing our robustness target are tensile strength, elongation at break, and energy at break (5).

Film expertise is critical for development of robust single-use bioprocessing bag solutions. Robustness comes from multiple properties such as film strength, film flexibility, seal-strength resistance, film weldability, and material puncture or tear resistance. So we characterized S80 film with regard to its tensile strength, elongation at break, energy at break, and so on compared with films used for other single-use bioprocessing bags on the market.

Expressed in Newtons (N), **tensile strength** describes the force required to break a film. Tensile-strength data are expressed in machine direction and transversal direction to take into consideration the manufacturing process influence. This specifically characterizes the film strength required for large-volume bags to resist hydrostatic pressure. When compared with other PE films, S80 film structure shows the best strength (Figure 3).

Expressed in percentage form, **elongation at break** is the maximum elongation that a film can withstand before breaking. These data represent the behavior of a film with regard to deformation and resistance to breakage. A high value for elongation at break characterizes a highly flexible film that resists to material fatigue. High resistance to material fatigue is required, for example, by bags used in shipping applications or in rocking-motion cell culture applications involving movement of liquid. Compared with other PE films, S80 film structure shows the highest value of elongation at break (Figure 4).

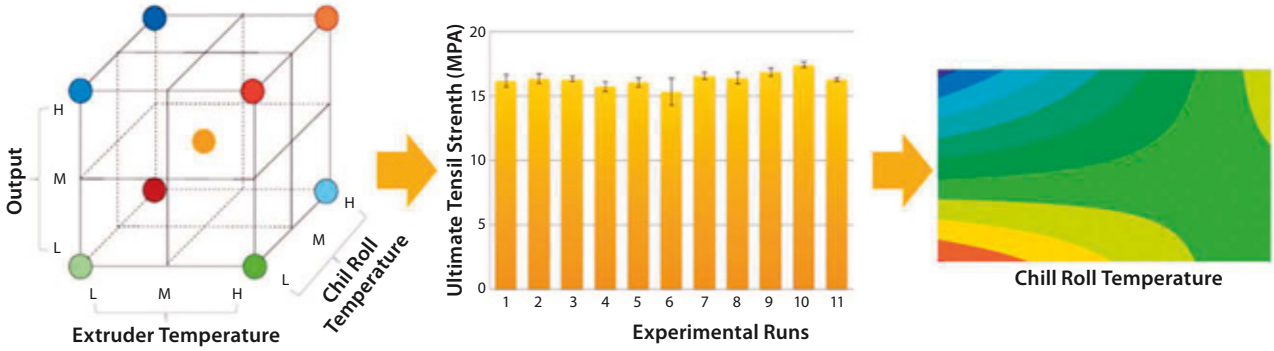
Expressed in Joules (J), the **energy at break** is the total energy required to break a film. This combines the maximum strength and maximum flexibility of the film in one direct measurement. It can be modeled by integrating the area under a strength/elongation curve to characterize the general robustness of a film. Compared with other PE films, S80 film structure shows the best energy at break (Figure 5).

Experiment: Once the best film structure has been identified, film-extrusion expertise is also critical to ensuring lot-to-lot consistency of CQAs and reproducible robustness of a film. This is achieved by identifying and controlling the critical process parameters (CPPs) of the extrusion process. During development of the extrusion process, the team explored process variability in a multivariate design of experiment (DoE). As a criteria for robustness, the ultimate tensile strength of prototype films was measured and shown to be consistent throughout an investigated range of parameters evaluated in the DoE (Figure 6). As for cell growth, we were able to define a design space for the extrusion process that provides assurance of achieving reproducibly the desired robustness for our S80 film.

The DoE allows for creation of a model to predict results depending on the CPP settings. The contour plot shows the ultimate tensile strength in machine direction (MD), when the chill-roll parameter is kept constant but output and extrusion temperatures vary within a given range. The contour plot illustrates the design space in which the predefined specification is achieved.

As a result of our QbD approach, S80 film used in Flexsafe bags is composed of a PE contact layer and a coextruded PE-EVOH-PE backbone structure (Figure 7). The combined strength and flexibility of each layer with a total thickness of 400 µm provides extraordinary overall robustness. The resin-additive formulations for both the contact layer and backbone are completely known and controlled according to specifications. Furthermore, the extrusion process parameters are

Figure 6: Example of a DoE experiment (using MODDE software) on film-extrusion design space with the ultimate tensile strength as a criterion



controlled within established ranges. This ensures long-term lot-to-lot consistency for all targeted critical mechanical quality attributes of Flexsafe bags — specifically, strength, flexibility, and (hence) robustness.

Welding and Bag-Making Expertise

The third critical expertise required for achieving robustness of Flexsafe single-use bioprocessing bags is in welding, sealing, and bag-making. The challenge is to define appropriate CPPs for consistently achieving the defined CQAs with a robust process. Our bag-making experts have optimized the welding, sealing, and bag manufacturing process and qualified these parameters based on a process risk analysis. Bringing together the knowledge of welding experts, application scientists, and production engineers ensures an adequate level of process understanding of the bag assembly and welding process.

CPPs for bag welding and sealing such as temperature and time have been considered in our QbD approach. Operating ranges of the welding parameters have been tested and are routinely controlled to ensure process consistency. To confirm robustness, especially of critical welds, we have established a water-burst test in which model bags are filled with water in a controlled manner. Thus, when evaluating sample bags, we found that typically our bags break at a certain overfilling level at the film rather than the welds (Figure 8).

Proven Robustness

The performance robustness of Flexsafe bags in different bioprocess steps in biopharmaceutical manufacturing has been confirmed against stringent applications such as cell culture, liquid shipping, long-term storage, and freezing applications. Robustness trials were conducted to test the performance of the product at its limit. As part of our film development, extensive robustness trials were performed under worst-case application conditions with rocking-motion bioreactor bags (100-L working volume) and stirred bioreactor bags (2,000-L working volume). Typically, these bags were filled with water to maximum their working volume. The bags were either rocked at maximum rocking rate or stirred at maximum stirrer rate for 21 days at 40 °C and maximum operating pressure. Weber et al. further explain this approach elsewhere in this special issue (7).

In addition, Flexsafe bags were tested for their robustness in air and truck shipping applications by applying the very stringent ASTM D4169-09 guideline for shipping applications (6). The testing sequence is designed to reflect worst-case conditions for air or truck shipment and performed at several temperatures. In addition to the handling test, the bags' truck-transport behavior is tested through investigating horizontal impact (Figure 9A) or rotational shocks. Air-shipping behavior is tested by exposing a shipper to vibrations (Figure 9B), compression, or a low-pressurized environment.

Our new Flexsafe bags successfully passed the most stringent ASTM D4169 test for air and truck shipment (Figure 9).

Figure 7: PE S80 film structure of the new Flexsafe bag family

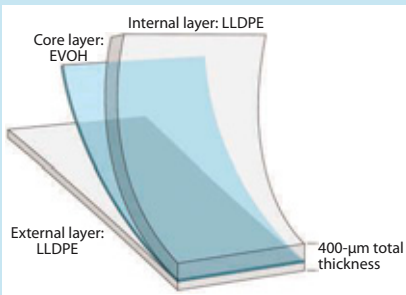
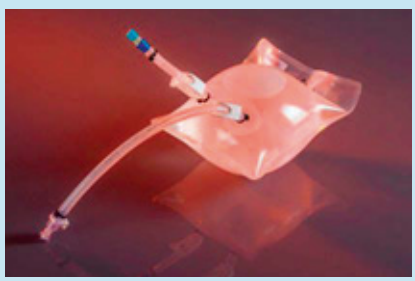


Figure 8: In-house water-burst test used to qualify the strength of bags and welds



Conclusion

With the increased implementation of single-use bags in biopharmaceutical drug production, there is a growing requirement for robust and easy-to-handle bioprocessing bags that will be suitable and scalable for all processing steps in upstream, downstream, and final-filling operations.

In partnership with our resin and film suppliers, we combined material science and film and bag-making expertise, applying the principles of QbD to select the best raw materials, optimize the design space of the extrusion process, and define the critical welding and overall bag making parameters. With this approach, our film experts designed several film formulations and combinations and were able to achieve

Figure 9A: Horizontal impact ASTM D880-92, method B

Figure 9B: Vehicle vibration ASTM D4728-06, method A (air spectrum)

Table 1: Strength and flexibility of film material and welds qualified using multiple methods

Qualification Test	Proven Performance
Standard flex durability of film	High resistance to fatigue and pinhole formation during handling, shipping, rocking, and mixing
Tensile strength of film and welds	High strength of film and welds; high resistance of bags to breakage under hydrostatic and air pressure
Elongation and energy at break	High flexibility and resistance to fatigue; ease of installation and use
ASTM D-4169-09	Film, weld, and bag robustness; safe and robust air and truck liquid shipping
In-house water-burst test	Strength of film, welds, and overall bag
Extensive worst-case testing	Robustness for stirred-tank and rocking-motion bioreactor bags and shipping applications

a new 400-µm thick film structure composed of a PE contact layer and a coextruded PE-EVOH-PE backbone that combine strength and flexibility. This optimal combination makes our new S80 film the most robust and suitable for all bioprocessing applications. The strength significantly reduces the risk of bag damage due to inappropriate handling, and the flexibility provides ease of installation and self-deployment of bags in their containers.

The formulation of resins and additives are completely known and controlled by specifications, and the extrusion process and welding parameters (e.g., temperature and time) are controlled within established process parameter ranges. This ensures long-term lot-to-lot consistency of strength, flexibility, and (hence) robustness of Flexsafe bags.

The superior robustness of Flexsafe bags was first demonstrated using standard flex durability, tensile strength, elongation, and energy-at-break testing. The S80 film structure shows the best strength, the highest value of elongation at break, and the best energy at break when compared with other PE films.

Mechanical property requirements largely vary by application. Rocking motion in cell culture or liquid-shipping applications requires a very flexible film that will resist material fatigue, whereas large-scale mixing applications with significant hydrostatic pressure call for strength in both film and welds.

That is why we have also applied a water-burst test developed in house and worst-case application testing of rocking-motion cell culture and stirred bioreactor bags. We confirmed the extraordinary robustness of Flexsafe bags in various applications and at maximum operating pressure, working volume, and rocking or stirrer rate over 21 days at 40 °C.

Finally, Flexsafe bags have been tested for robustness in shipping by both air and truck, applying the very stringent ASTM D4169-09 guideline (designed to reflect worst-case conditions for air or truck shipment) at several temperatures.

Acknowledgments

Thanks to Sartorius Stedim R&D teams in Aubagne, France and Göttingen, Germany.

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Enhanced Assurance of Supply for Single-Use Bags

Based on Material Science, Quality By Design, and Partnership with Suppliers

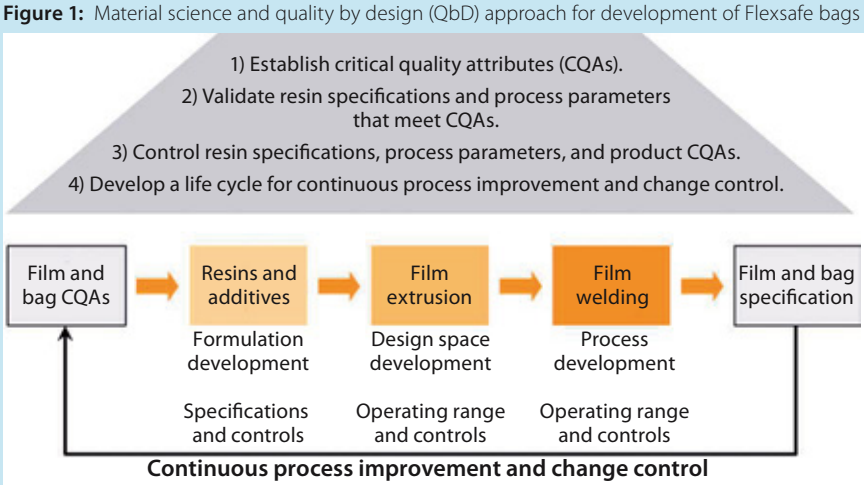
by Jean Marc Cappia, Elisabeth Vachette, Carole Langlois, Magali Barbaroux, and Heiko Hackel

Growing adoption of single-use bags in commercial production of biopharmaceutical drugs raises new challenges for bag suppliers and drives the need for consistent product quality, improved assurance of supply, robust change management, and business continuity planning. In close collaboration with resin and film suppliers, polymer scientists and biologists at Sartorius Stedim Biotech have followed a stringent material science and quality by design (QbD) program to develop a completely new polyethylene film and to achieve consistent performance of new Flexsafe bags for all bioprocess steps and applications.

Reliability of the supply chain for Flexsafe bags is based on partnerships and agreements with suppliers and our complete understanding and control of the bag manufacturing process — from resin and film extrusion to final sterile bioprocessing bags. Consistent product performance is ensured by establishing specifications, traceability, and control of resins and additives as well as definition of a design space for film extrusion.

Assurance of supply relies on a long-term contract and partnership with our film supplier and its large extrusion capacity, which allows us to absorb the projected double-digit growth for single-use solutions. In addition, establishment of resin specifications rather than simply using resins based on their trade names provides robust change control and facilitates change management. Our company can rapidly qualify equivalent resins sourced from preferred resin suppliers should one supplier discontinue or introduce changes to necessary raw materials. Business-continuity planning is finally addressed through a mix of already available redundant equipment and manufacturing locations, qualification of back-up equipment, and maintenance of safety stocks for resins and film rolls.

Figure 1: Material science and quality by design (QbD) approach for development of Flexsafe bags



Facing New Challenges

Single-use bioprocessing bags are widely adopted today by established biopharmaceutical companies for development and commercial production of biological drug products and vaccines. Single-use technologies offer safer, cheaper, faster, smaller, and "greener" biomanufacturing options and meet the main challenges of the biopharmaceutical industry for capacity adaptation, cost savings, risk mitigation, and process transfer. For smaller biotechnology companies, single-use technology also offers a fast and economic approach to rapidly implement flexible manufacturing capacity (**1**).

The technical and economic benefits of using single-use technologies are well known, and the biotechnology industry is broadening their adoption into all bioprocess steps such as cell culture, storage, shipping,

mixing, freezing and thawing, final filling, and sampling (2). In the past, most of the single-use bags were used for noncritical media and buffer storage applications. Those bags did not necessarily address fully the more critical quality demands of biopharmaceutical process intermediates, bulk drug substances, and final drug products.

The growing use of disposable bags in all critical process steps — both for process development and commercial production of high-quality biological drugs — raises new challenges for bag suppliers. Modern single-use solutions need improved quality, robustness and assurance of supply; more robust change control; and well-established business continuity plans (3).

A Partnership, Material Science, and QbD Approach

To meet those new challenges for a more robust quality of single-use bags, Sartorius Stedim Biotech developed a new, proprietary, PE S80 film in partnership with Südpack, our film manufacturer. That new film is the core component of our new Flexsafe family of single-use bags for all bioprocess applications. Through our collaboration with Südpack, we have established direct contacts, trust, and long-term relationships with selected industry-leading suppliers of plastic resins. Direct access to (and the openness on the part of) our raw-material suppliers provides an unprecedented level of information on the initial resin and additive package formulation. That is the central part of our new strategy to achieve complete control over our entire supply chain, from raw materials to finished products. Our resin suppliers support our assurance-of-supply strategy through their open mind-set and strong understanding about (and contribution to) risk-analysis and change-control concepts.

The partnership with Südpack, open attitude, and long-term relationship with our resin suppliers have allowed us to combine material science and QbD expertise to achieve a complete understanding and control of our manufacturing process from the initial raw materials to the final bioprocessing bags.

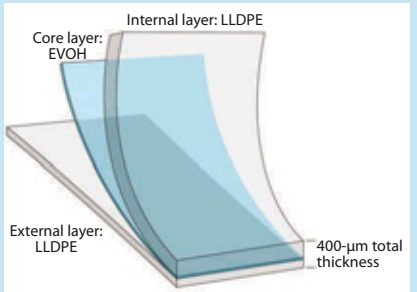
We selected our resin–additive package and processing parameters for the S80 film after testing several raw materials and film compositions to identify one with the best physical and mechanical properties and the most consistent cell growth performance (5, 6). The film specification was established to enable reproducible manufacturing of Flexsafe bags that are suitable and scalable for all upstream, downstream, and aseptic bioprocessing steps, including cell culture, storage, shipping, mixing, freezing, thawing, and filling applications. Key user requirements cover, for example, consistent cell growth, biocompatibility, purity, robustness, gas barrier properties, cleanliness, and sterility of final single-use Flexsafe bags.

The biocompatibility of films is normally assessed using conventional USP chapter <87> and chapter <88> in vitro and in vivo biological reactivity tests. For the new Flexsafe bags, a standardized cell-growth assay was used at the design stage to optimize the resin, the additive package, and the film formulation and to ensure their compatibility with a broad range of cell lines (5). Cell-growth testing also allowed us to determine the operating ranges for extrusion, welding, and γ-irradiation processes and establish specifications and critical process controls (4).

Purity of films and bags is characterized by their extractable and leachable profiles. It is determined by the nature of the polymer, the amount and nature of additives used to enable processing, and the processing parameters themselves. With a well-known and well-controlled resin, our polymer experts can thoroughly characterize the extractable profiles of Flexsafe bags and ensure their reproducibility. End users can be assured that their own initial extractable and leachable qualification work and data will remain valid every time they operate single-use bioprocesses using Flexsafe bags.

The robustness and barrier properties of Flexsafe bags are obtained by combining thickness, strength, and flexibility for each layer of S80 film. Together with application knowledge, our resin, film, and bag expertise allowed us to develop a 400-µm thick structure that offers outstanding robustness of film, welds, and bags for all bioprocessing applications in upstream production, downstream processing, and final filling.

Figure 2: PE S80 multilayer film structure of the new Flexsafe bag family (patent pending)



The S80 film used in Flexsafe bags comprises a polyethylene (PE) contact layer and a coextruded backbone structure of PE, ethylene vinyl alcohol (EVOH), and PE. The formulation of resins and additives for both the contact and backbone layers are completely known, and the extrusion process parameters are controlled within established process-parameter ranges. That ensures lot-to-lot consistency of all critical quality attributes (CQAs) of Flexsafe bags: cell growth, robustness, barrier properties, and extractable profiles.

Enhanced Quality Assurance and Assurance of Supply

The entire supply chain for single-use bags is complex and requires material science and film expertise to achieve consistent quality attributes of film; bags; and final, sterile, single-use assemblies.

First, the key aspect of quality assurance consists in collaboration with manufacturers of resins and additive packages used for extrusion of polymer films. Working directly with suppliers of raw materials and plastic resins is of paramount importance in establishing specifications, operating ranges, and process controls and obtaining full understanding and traceability of the initial resin and additive package used for making a film.

Figure 3: Overall supply chain for single-use bioprocessing bags

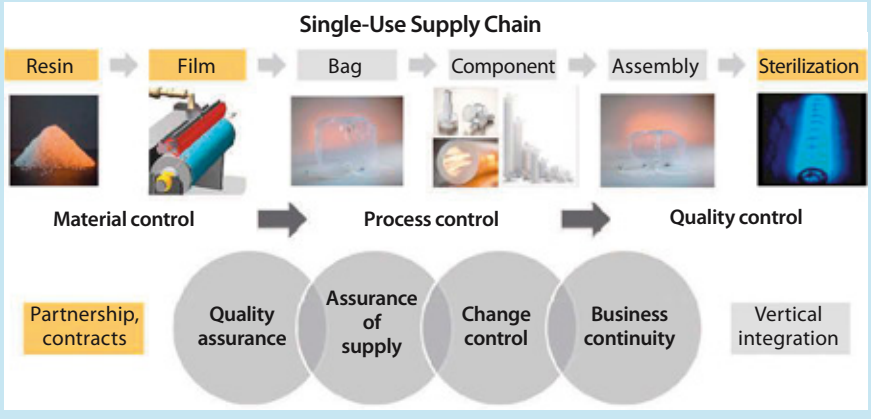
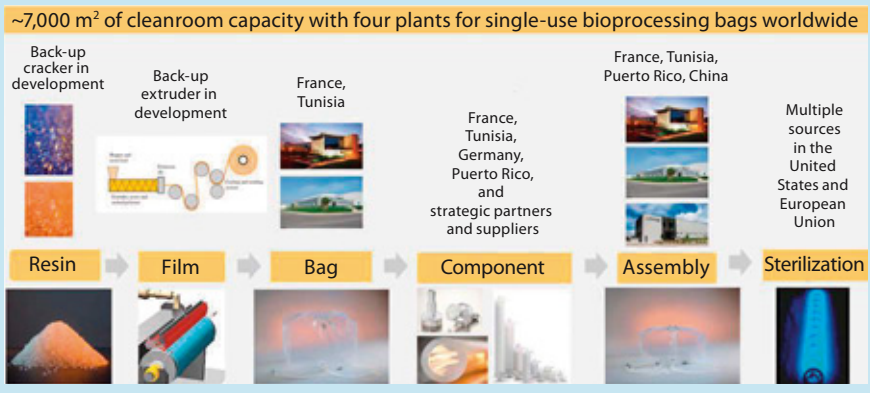


Figure 4: Global Sartorius Stedim Biotech supply chain and manufacturing infrastructure for single-use bags



Second, robust product quality necessitates a full understanding of the complete process, definition of operating ranges, and implementation of multiple controls to guarantee lot-to-lot performance of films, bags, and single-use bag assemblies. Such an overall knowledge and understanding of the entire process — from resin to final product — can be achieved only through strong relationships and partnerships among single-use bag manufacturers, raw-material suppliers, and film manufacturers.

Our knowledge about the formulation of the resins, additives, and catalysts as well as the critical process parameters (CPPs) for film extrusion makes it possible for us to understand which CPPs influence the CQAs of our final product. This QbD approach helped us develop more detailed resin specifications for both the contact layer and film backbone.

Also, our detailed knowledge regarding the formulation of compounds used in manufacturing our films enables us to establish consistent and relevant extractable profiling, data reporting, and leachable validation support. Knowing the compounds in the films helps us interpret our extractable data very precisely and accurately. This approach has been implemented to consolidate the supply of our existing S71 ethylene vinyl acetate (EVA) film used for making Flexboy and Celsius bags and in development of our new S80 PE film used for the new Flexsafe bag family.

Assurance of Supply Through Partnership and Long-Term Supply Agreements with Resin and Film Suppliers: Supply assurance for Flexsafe bags is guaranteed first by our long-term supply contracts established with different resin suppliers and with Südpack, our film manufacturer. We have a 10-year supply agreement in place for the S80 PE film and a last-time buy option for a minimum of two years of resin demand in case of changes or discontinuation. We also benefit from Südpack's large, state-of-the-art, film-extrusion capacity, which will be required to sustain an expected yearly double-digit growth of the demand for single-use bioprocessing bags. Here again, complete control of our supply chain and manufacturing processes — from raw materials to final assemblies — is critical to offering both quality and assurance of supply.

Robust Change Control Procedures

Our long-term contracts with resin and film suppliers involve six months' notification for changes in raw materials and/or manufacturing processes of the resin and two years' notification for changes to the film. In addition, we have a last-buy time option of unchanged material for meeting two years of resin demand.

Even more important is establishment of resin specifications instead of using resins by their trade names. This provides robust resin change control and facilitates change management. With our established specifications and understanding of the resin's CQAs, we can rapidly qualify an equivalent resin with our preferred resin suppliers. Releasing and controlling resins against specifications — not trade names — enhances the reliability of film quality, facilitates change management, and improves long-term assurance of supply with fast implementation of alternative resins in case of change or discontinuation of some raw materials. And the design space we have established for the film-extrusion process provides a fast and easily qualified implementation of new extrusion equipment should a change or a capacity ramp-up be required.

Business Continuity Planning

The terms *business continuity planning* and *assurance of supply* often, incorrectly, are used interchangeably.

Assurance of supply reflects our capability as a leading single-use supplier to consistently provide products according to defined specifications and quality requirements under normal operating conditions. This is the result of our long-term contracts, our large manufacturing capacity for all process

steps, and the control of our entire process from raw materials and films to final sterile products.

Business continuity planning is required to cover unexpected and unlikely events such as raw-material discontinuation or manufacturing shut-down due to earthquake, fire, flood, storms, or other potential disasters. This is best achieved by ensuring that every critical process step can be performed with at least two different pieces of equipment and/or at two different manufacturing locations. When neither redundant process equipment nor an alternative production site is available, another alternative is to maintain safety stocks that cover demand for the time that would be necessary to build and qualify alternative equipment or a new production line.

Sartorius Stedim Biotech is currently qualifying a backup resin cracker and a second film extruder at our suppliers and partners to further enhance our business continuity planning. In the meantime, we maintain at least two years of safety stock for resins and films. Multiple bag-making equipment and assembly lines are installed in multiple manufacturing locations in France, Tunisia, and Puerto Rico to ensure business continuity planning for our final bag assemblies. We also have contracted multiple gamma-irradiation sterilization sources and suppliers worldwide to ensure final sterility assurance.

Table 1: Global approach to quality, assurance of supply, change control, and business continuity

Challenges	Solutions	Results
Consistent quality	Relationships with resin suppliers Partnership with film manufacturer	Specifications, traceability, and control of resin and additive package ensure resin lot consistency. Design space and control of extrusion parameters ensure film lot consistency.
Assurance of supply	Contracts with resin suppliers Supply contract with film manufacturer	10-year contract with film manufacturer Last-time buy clause for minimum of two years of resin demand Large extrusion capacity aligned with growing demand Control of complete resin-to-bag process
Change control	Partnership and contracts with suppliers Specifications and design space for resin	Six-month notification for resin change Two-year notification for film change Resin specification and extrusion design space provide for fast implementation of alternative resin/film.
Business continuity	Back-up resin crackers and film extruder in development Safety stocks of resins and films Multiple bag manufacturing locations and γ-sterilization contractors	Future redudant resin and film processes Two years of resin and film safety stock available Capability to balance capacity between multiple sites in Europe, Tunisia, Puerto Rico, and China

Conclusion

Sartorius Stedim Biotech has been working over the past few years on a strategy to enhance quality, supply assurance, and change control for its single-use bioprocessing bags and intelligent single-use solutions consisting of bags, filters, tubes, connectors, sensors, automation, and hardware. This strategy is based first on partnerships and long-term contracts with suppliers, which ensure control of raw materials and films as well as extension of resin and film-extrusion capacity. Second, it is based on the continuous expansion of our own manufacturing capacity in multiple sites to allow flexible business contingency planning. This strategy has been applied both in development of our new S80 polyethylene film and Flexsafe bag family and in strengthening the supply chain of our existing S71 film made of an EVA contact layer and used for manufacturing Flexboy and Celsius bags.

Establishing resin specifications and film-extrusion design space for controlling the entire manufacturing process provide long-term supply assurance, consistent product performance, and robust change control. Flexible business continuity planning is guaranteed by establishing back-up equipment for resin cracking and film extrusion, multiple bag manufacturing locations, and safety stocks of resins and extruded film rolls. In addition to the current EVA and new PE bags, we also control the manufacture of other critical components such as filters, connectors, tubing, and sensors and assemble them into our single-use solutions at multiple manufacturing locations worldwide.

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Development and Qualification of a Scalable, Disposable Bioreactor for GMP-Compliant Cell Culture

by Anne Weber, Davy De Wilde, Sébastien Chaussin, Thorsten Adams, Susanne Gerighausen, Gerhard Greller, and Christel Fenge

During the development of single-use, stirred-tank bioreactors (e.g., BIOSTAT STR bioreactors), different phases can be distinguished (Figure 1). First, a clear definition of the intended application and all related requirements should be captured in a user requirement specification (URS). Based on that, the single-use bioreactor design phase and the material selection phase are initiated, both closely linked to each other. During the proof-of-concept phase, relevant component- and product-based tests are established and realized to ensure URS compliance. Finally, the qualification can be divided into product and process qualification, in which the latter includes qualification of the production equipment procedures and parameters. This process, together with stringent change-control procedures, will positively influence batch-to-batch consistency and security of supply.

Final product qualification should include qualifying the product's individual components and the final product using mechanical and biological tests emulating the intended application or a combination of both. Those tests should be performed on bioreactor bags derived from regular manufacturing.

Figure 1: Phases during product development of a single-use bioreactor

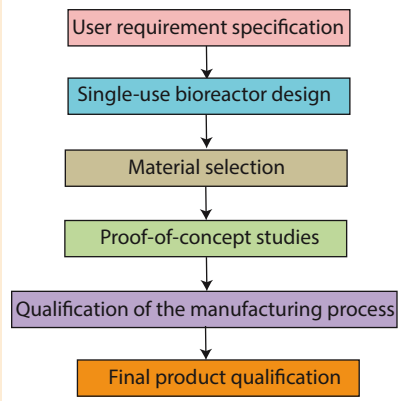


Photo 1: BIOSTAT STR 2000 bioreactor

User Requirement Specification

At the start of BIOSTAT STR bioreactor development, key user requirements are defined covering basic requirements of cell growth and productivity, robustness, reliability, and security of supply. Leachables from plastic materials should be considered to ensure a reproducible biological performance because such substances can affect cell growth (**1**). Other key requirements are proper mixing and oxygen transfer (**2**). The stirrer design should allow for superior mixing while minimizing shear forces.

Stainless steel processes typically include highly automated cleaning and sterilization. Single-use bioreactor set-up and change-over require a higher level of manual handling. Therefore, bag handling must be considered during the development process to make it easy-to-use and limit risks for misuse leading to loss of bag integrity.

Single-Use Bioreactor Design

Because the characteristics of stirred stainless steel bioreactors are well understood, this culture vessel design has been the gold standard for bioprocessing for decades. Based on that, the BIOSTAT STR single-use bioreactor family (Photo 1) was developed and designed from

12.5 L to 2,000 L (**2**). A detailed description of the single-use bioreactor design is given by De Wilde et al. (**2**).

The BIOSTAT STR bioreactor is equipped with fluorescence-based single-use sensors for pO_2 and pH measurement and control. Sensor patches are installed and sterilized together with the cultivation bag. Modern single-use sensor technology ensures the same level of control as traditional sensors but avoids risky insertions of classical probes (**3**). The functionality of the probes is tested successfully by Reglin et al. (**4**) in a high–cell-density, 1,000 L STR fed-batch CHO cultivation using chemically defined media. Reusable probes can be inserted as an alternative if desired.

Material Selection

A single-use bioreactor bag mainly consists of various components made of polymer resins. Raw material selection for the individual components is guided by regulatory considerations, mechanical properties needed for the bioreactor's intended use, and the need for a biological performance comparable with glass and stainless steel bioreactors (**5**). We followed guidelines that are applicable to polymeric materials that come into contact with final pharmaceutical products (e.g., requirements established for pharmaceutical packaging). Guidelines related to the food and medical-devices industries also can facilitate assessment of the suitability of a material for bioprocess applications (**5**). Table 1 presents examples of relevant regulations that should be taken into account when qualifying raw materials of polymeric nature for single-use bioreactors.

The Flexsafe STR bag contains internal moving components (e.g., the agitation system) that are subject to substantial mechanical forces during bag manufacturing, transport, installation, and use. Therefore mechanical properties of the raw material (e.g., hardness and tensile modulus) must be carefully considered, not only with regard to component performance (e.g., tensile strength), but also for component manufacturing (e.g., melt flow index). Once the components are fabricated out of a selected raw material, they have to be evaluated, typically in the final assembly during the proof-of-concept phase.

Table 1: Examples of relevant guidelines

	Reference	Title
Biocompatibility	ISO 10993-5	*Biological Evaluation of Medical Devices: In Vitro Cytotoxicity*
	US Pharmacopeia <87>	*Biological Reactivity Test, In Vitro*
	US Pharmacopeia <88>	*Biological Reactivity Test, In Vivo*
TSE/BSE	European Medicine Agency A410_01_re2 or *European Pharmacopoeia* 5.2.8	*Minimizing the Risk of Transmitting Animal Spongiform Encephalopathy Agents via Human and Veterinary Medicinal Products*

Proof-of-Concept Studies and Production Process Qualification

A key element in biopharmaceutical development is transfer of a cultivation process from laboratory to production scale while ensuring comparable process characteristics (**6**). Although a design-space approach can support successful process transfer (**7, 8**), scale-up remains a challenge and is "as much an art as a science" (**9**). Therefore, extensive process understanding and knowledge of the bioreactors and equipment in use is a prerequisite (**10**).

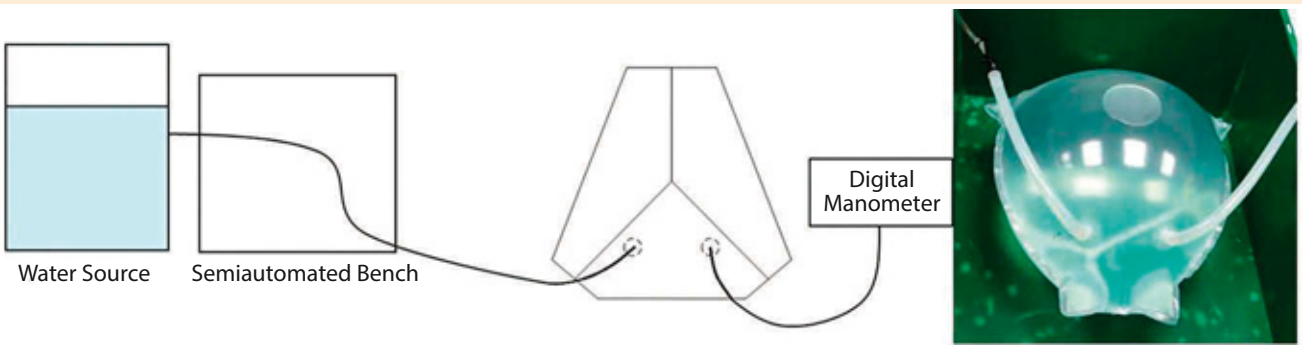
During the proof-of-concept phase, the design space with regard to agitation and aeration rate was determined, and a system characterization was conducted, in

Table 2: Example approach to qualify and characterize a film for bioreactor applications

Film Properties	Objective	Tests Performed
Biocompatibility	To guarantee a biocompatible film (including overall materials and manufacturing process)	In accordance with the criteria of USP<87> Biological Reactivity Test, In Vitro; USP<88> Biological Reactivity Test, In Vivo; ISO10993-5 Biological Evaluation of Medical Devices, In Vitro Cytotoxicity; and in-house cell culture trials
Robustness	Evaluation and qualification of mechanical properties required during the applications	During a single-use bioreactor application the film is exposed to puncture, traction, flexion, and fatigue (Photo 2). In accordance with ISO527-3 or ASTM D 882, tensile test allows characterizing the tensile properties of the film and the ability to resist to the elongation. In accordance with ASTM D 3787 (an adaption), ball bursting allows characterizing the ability to resist puncturing of nonsharp items through the film. And in accordance with ASTM F 392 condition C, flex durability test allows characterizing the ability of the film to resist to flexion and fatigue in flexion.
Interaction	Evaluation of physical–chemical properties	In accordance with the criteria of USP<661> for aqueous extraction for nonvolatile residue, residue of ignition, heavy metals, and buffering capacity
	To guarantee compliance of the contact layer composition with the *European Pharmacopoeia* and to evaluate physical–chemical properties of the contact layer	In accordance with the criteria of *European Pharmacopoeia* 3.1.5: for polyethylene with additives for containers for parenteral preparations and for ophthalmic preparation
	Characterization of the extractables and leachables profile of film including an evaluation of interaction with the properties of water for injection	In accordance with a Sartorius model: Identification and semiquantification of volatile compounds, semivolatiles compounds, nonvolatiles compounds and metal elements extracted/leached from the film; representative solvents are used to create the extractable guide; characterization of the pH shift, conductivity, total organic carbon (TOC) and nonvolatile residue (NVR) of water for injection stored in bags made with the film

Photo 2: Tensile and flex durability tests (photos left and middle from Reference **19**)

Figure 2: Water burst test with a filled bag chamber



which typical scale-up parameters were evaluated (**11**). De Wilde et al. describe a detailed characterization approach (**2**). And Reglin et al. (**4**) describe a successful transfer of a high–cell-density CHO fed-batch process to a Flexsafe STR 1000 bioreactor finalizing the proof-of-concept phase.

After a successful proof of concept, the final design was transferred into production, including operator training and qualification of the production equipment and procedures. Production parameters were monitored, and requalification of them was performed in regular intervals. A stringent change-control process ensures consistent quality, performance, and reliability.

As part of ensuring a high level of supply assurance, current regulatory guidelines and standards for quality systems such as ISO 9001 (**12**) were followed. These guidelines instruct manufactures of single-use bioreactors to control components and services obtained from subsuppliers. During supplier qualification, their quality system, their experience in offering materials for pharmaceutical or medical applications, and the suppliers reliability are critically reviewed. Suppliers of raw materials for components in direct contact with process fluids should apply a quality system meeting international standards such as ISO 9001, ISO 13485, or CFR 21 Part 820 (**5**). Finally, specific design parameters (e.g., critical dimensions and component delivery times) are established for all critical parts.

Final Qualification Approach

To perform a proper qualification, it is not sufficient to focus only on the qualification of the components and the manufacturing process. Qualification of the final product under relevant conditions for the intended use is absolutely critical.

By contrast with pharmaceutical plastic packaging, no regulatory guidelines have been specifically developed for the qualification of single-use bioreactors. Therefore, the qualification approach of STR bags is based on general good manufacturing practice (GMP) principles and relevant parts of existing guidelines associated with other products. For the component-based qualification approach, test procedures for individual components are implemented. The qualification approach for the entire final product is based on the verification of the materials and assemblies during the performance qualification at the application level. Therefore, a combined approach of qualifying individual components as well as qualification at application level of the final product is considered the most meaningful and effective way to qualify such a complex system (**2**).

Selecting and Qualifying the Proper Film Properties

Films used in bioprocess applications commonly possess a multilayer structure. Currently marketed films include a backbone and a contact layer. The backbone provides the barrier structure and consists of one or more layers, which determine the overall mechanical behavior and barrier properties. The contact layer is the surface that comes into contact with culture broth. It must be inert and have good sealing characteristics (**5**).

Examples for properties typically tested in accordance with regulations applicable to biopharmaceutical packaging are shown in Table 2. Some of those tests are defined to qualify the film with pass–fail criteria. Others are performed to characterize the film, without specifications.

The influence of substances leached from the film on the growth and

Figure 3: Pressure plot obtained during a robustness trial of a Flexsafe STR 1000, including final aeration and pressure stress test

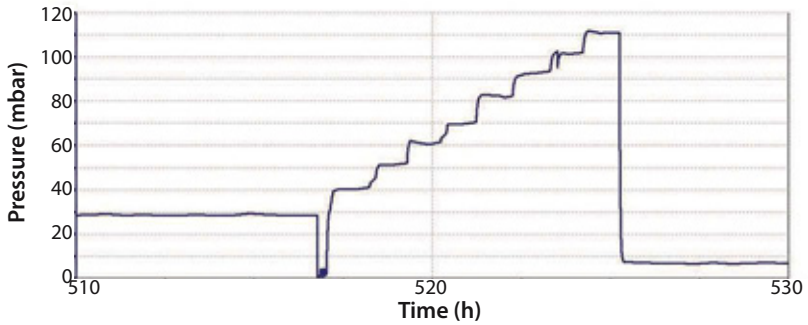
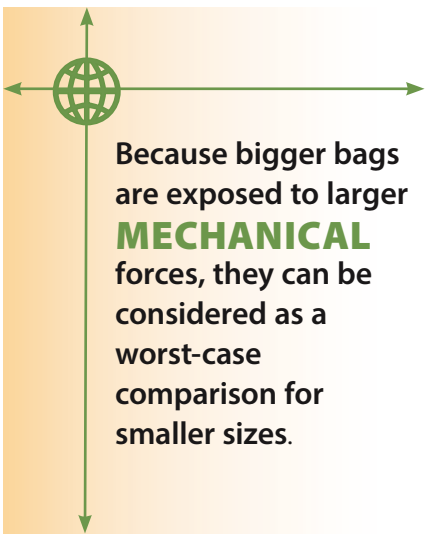


Table 3: Assessment of the robustness trial

	Step	General Acceptance Criteria
Before installation	Unpacking	No damages to the packaging
	Visual inspection of the bag	No film or component defect No damages to welds, inner components, film, ports
	Installation of the bag in the bag holder	No deviations to the steps in the BIOSTAT STR manual
Final visual inspection	Agitation trial	No bag damage
	Aeration trial	No internal component/stirrer defect as per visual inspection from outside the bag Functionality of the agitation system Functionality of the aeration system No external component defect



productivity of mammalian cells has been identified as one of the major concerns related to the use of single-use bioreactors. Fenge et al. (**1**) summarize the approach to investigate biocompatibility of the new S80 polyethylene film used in the Flexsafe product range.

A design space (process window) for critical process parameters of film extrusion was defined to

• guarantee extrusion process reproducibility within established parameters settings of the design space

• guarantee film quality and reproducibility

• guarantee that film properties reach the acceptance criteria when films are produced within the design space.

The properties of the film were also verified after aging it three years to establish the film's shelf life. Both accelerated aging regulated by ASTM F 1980 (**13**) and natural aging conditions were considered to qualify the three-year shelf life of the film.

Qualification of Assemblies

The various connections between individual components of the single-use bioreactor were qualified to demonstrate physical and mechanical integrity of the final bag assembly. To demonstrate reliability, reproducibility, and robustness, seal strength and integrity were qualified. Robustness was qualified both on final-product level and on component level.

A water-burst test (Figure 2) was developed to evaluate the robustness of critical welds. A certain nominal volume could be reached before bursting — caused by a breakage of the film rather than at the welds — of the model S80 bag (**14**). Comparing these results with those obtained from other commercially available films (not shown) confirms superior robustness of the new S80 polyethylene film used for the manufacturing of Flexsafe bags. For the conversion of the STR bag family to the new S80 film, each weld of the single-use bioreactor chamber was subject to tensile strengths, color invasion, and water-burst testing. The color invasion test allows identification of the presence of microchannels in a weld. Different film lots were used to conduct those tests.

Successful Realization of the Final Bioreactor Product

Ensuring State-of-the-Art Bioreactor Bag Robustness: Qualification of the STR bags with S80 film was based on a worst-case rationale relevant to the final application. Because bigger bags are exposed to larger mechanical forces, they can be considered as a worst case comparison for smaller sizes. Three film lots were used for the final qualification approach, and a representative bag quantity was tested. Additional robustness trials will be performed routinely on bags from regular production to confirm the results of the initial qualification.

The parameters of the robustness trial were chosen based on the typical characteristics of a mammalian cell-culture process. In the 21-day trial, a maximum filling volume, maximum stirrer speed, 40 °C, and 30 mbar overpressure were applied. Additionally, an aeration test and a pressure stress test were conducted following the 21-day period.

During robustness qualification and routine testing, a pressure of up to 50 mbar was evaluated. Additionally, a worst-case pressure stress test on a selected number of single-use bioreactor bags was performed. That consisted of increasing the pressure from initially 30 mbar to 110 mbar in 10-mbar steps (Figure 3). Every pressure-step increase was maintained constant for one hour. Before and after the robustness trial, a visual inspection

Table 4: Stability of water for injection in accordance with the European and US pharmacopoeias

	Reference	Title
Total organic carbon (TOC)	*US Pharmacopeia* <643>, European/US Pharmacopoeia Monograph for "Sterile Water for Injections"	*Total Organic Carbon*
Conductivity	*US Pharmacopeia* <645>; European/US Pharmacopoeia Monograph for "Sterile Water for Injections"	*Conductivity*
pH	*US Pharmacopeia* <791>; European/US Pharmacopoea Monograph for "Sterile Water for Injections"	*pH Determination*
Ions and oxidizables	European/US Pharmacopoeia Monograph for "Sterile Water for Injections"	*Stability of Sterile Water for Injection*
Nonvolatile residue (NVR)	*US Pharmacopeia* <661>; European/US Pharmacopoeia Monograph for "Sterile Water for Injections"	*Polyethylene Container*
Particulate matter	US Pharmacopeia <788> and European Pharmacopoeia 2.9.19	*Nonvisible Particles*

of the whole bag, seams, and the agitation system was performed. Table 3 lists the defined pass criteria for the robustness trials.

Sterilization Validation to Ensure Risk-Free Cell Cultivation: The Flexsafe STR system is sterilized by gamma irradiation. The first step in the validation of a gamma sterilization cycle with a security assurance level (SAL) of 10^{-6} described by the ISO 11137 (**15**) is assessment of the final bag product's bioburden load.

Determination of the bioburden of the Flexsafe STR family is based on an in-house method in accordance to ISO 11737 (*Sterilization of Medical Devices: Microbiological Methods, Part 1 — Determination of a Population of Microorganisms on Products*) (**16**).

A dose-mapping study of the Flexsafe STR product family was performed according to ISO regulations (**15**) and based on a worst-case rational. Because it is directly linked to product density, including its packaging, we defined the packaging and performed a transportation test (described below) before dose mapping. The verification dose is calculated to achieve SAL 10^{-1} according to ISO 11137 (**15**). Individual sterility testing was performed on a specific number of STR bags.

Packaging Validation to Ensure Full Robustness in a World without Boundaries: Packaging was qualified by a transportation test simulation in accordance to ASTM D 4169-DC 2 (**17**) to assure robustness and protection against mechanical handling (impacts and drops), vehicle vibration (truck and aircraft cargo), and stacking. The Flexsafe STR 1,000-L unit was tested and was taken as the representative for the STR 500-L unit because of their nearly identical packaging dimensions. The Flexsafe STR 50-L and 200-L models were tested because they differ in terms of unit per pallet and height. Furthermore, the Flexsafe STR 2000-L unit was successfully tested as a worst-case configuration in terms of height and weight.

Stability of Water for Injection: Apart from the mentioned qualification approaches for robustness and sterility, leachable data are provided. The stability for water for injection is specified by the monographs of the US and European pharmacopoeias. Tests were applied to gamma-irradiated Flexsafe STR bags (Table 4) and documented in a validation guide. Results from the stability of water for injection are not considered as release criteria but serve as comparable data for the pharmaceutical industry because mammalian cell-culture media are used during the application.

For the stability of water-for-injection trials, three subfamilies are defined, considering the construction of the agitation system. Three bags per subfamily from three different film lots were tested. The conditions were 40 °C for 21 days with identical working volume, pressure, and agitation speed as for the robustness trial, comparable to a worst-case cell-culture application. In accordance with *US Pharmacopeia* <85>, endotoxin values were determined.

Extraction with Water and Ethanol: Extractables can directly influence the quality of a final product. Therefore, in each pharmaceutical production step, potential contamination with extractables has to be assessed. Detailed knowledge of the chemical nature of extractables is essential even for trace amounts.

The analytical scheme was developed in line with the concept that a bag is made up of a large number of different polymeric materials. Potential extractables could consist of respective breakdown products, residual monomers, and oligomers from manufacturing residues and additives of the different polymers. Such a broad spectrum of substances represents a complex analytical challenge that can be managed only by combining different analytical methods. Typically, various mass spectrometry methods are used and extractables are investigated, with the amount and nature determined.

For developing the analytical scheme, the two most commonly used solvents (reverse osmosis water and absolute ethanol) were chosen to create a database covering all extractables. For this study, a rational approach was used that considered the different components of the Flexsafe STR family and a worst-case ratio of film surface to volume. The extraction conditions (40 °C for 21 days) are considered to be comparable to a typical mammalian cell-culture process.

Long-Term Flexsafe Bag Stability: Shelf-life qualification of the Flexsafe STR bags is comparable to the verification of STR bags made of S40 film (**18**). Based on a risk assessment, the STR 2,000-L, 1,000-L, and 200-L models were chosen to determine robustness, stability of water for injection, and functionality of the optical sensors after accelerated ageing in accordance to ASTM F 1980 (**13**) and natural ageing after two years. Storage conditions for

accelerated aging are 225 days at 40 °C and 75% humidity. This correlates to two years natural aging.

Conclusions

Unlike other single-use bioreactor designs available on the market, the BIOSTAT STR bioreactor family is unique in its design and performance because its design is similar to that of conventional stainless steel stirred tank bioreactors. For development and qualification, specific tests were selected or developed to ensure full robustness, reliability, and lot-to-lot consistency. Robustness, cell culture performance, and quality of this disposable bioreactor family are based primarily on the selection of suitable polymer materials and a well-defined and controlled production process to ensure that critical quality attributes are consistently met. Furthermore, an intelligent, application-driven qualification approach both on component level as well as on final product level was established that emulates the intended application of the advanced single-use bioreactor platform. This ensures a holistic qualification approach starting from the raw materials to the final single-use bioprocessing bag product in its intended application.

Full functionality and consistent and reproducible performance could be confirmed, and biological compatibility was proven by mammalian cell-culture trials (**1, 4**). Superior and consistent robustness was confirmed in worst-case application trials and pressure tests. All relevant and recommended tests according to US and European pharmacopoeial monographs and an extractable study were conducted and passed successfully. Finally, qualification of the BIOSTAT STR single-use bioreactor family confirmed that the user-requirement specification (which was defined at the starting point of the product development) was consistently met.

The risk-based approach of testing at component level and on final-product level, in the intended application, ensured the successful development of a robust single-use bioreactor. That bioreactor is not only highly comparable with existing stainless-steel bioreactors, but also meets industry requirements with regard to quality, robustness, and assurance of supply.

Acknowledgment

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Verification of New Flexsafe STR Single-Use Bioreactor Bags

Using a CHO Fed-Batch Monoclonal Antibody Production Process at 1,000-L Scale

by Regina Reglin, Sebastian Ruhl, Jörg Weyand, Davy De Wilde, Ute Husemann, Gerhard Greller, and Christel Fenge

In the past decade, single-use bioreactors have gained wide acceptance for biomanufacturing. The biopharmaceutical industry is increasingly interested in performing modern production processes in single-use facilities. That trend is driven by the time and cost benefits of single-use technologies, as well as the enhanced manufacturing flexibility they offer (**1**).

With single-use bioreactors increasingly used in late-phase clinical trials and commercial production, their quality, reliability, and assurance of supply becomes more critical. Many industry experts consider process control of film and bag manufacturing and traceability of raw materials to be key to ensuring batch-to-batch consistency with single-use bioprocessing bags. Especially crucial for such bags is excellent and consistent cell-growth performance for a broad range of different commercially relevant cell lines.

Most commercially available film materials used for single-use bioreactors today were not specifically developed for cell culture applications. Therefore, Sartorius Stedim Biotech partnered with Südpack (Europe's leading film-extrusion company) and closely collaborated with resin and additive suppliers to develop a new polyethylene film ("S80") specifically optimized for such applications (**2, 3**). This collaborative concept enabled us to develop a completely new range of bioprocessing bags that meet industry needs for future biomanufacturing: consistent cell-growth and extractable profiles, superior robustness and ease of use, unprecedented assurance of supply, and suitability for all applications from upstream production to downstream processing and final filling (**3–5**).

The S80 polyethylene film used in Flexsafe bags has an optimized additive package controlled by specifications rather than trade names of resins and additives. Film extrusion, bag assembly, and γ-irradiation are all controlled within established process parameter ranges. This approach based on specifications and controls prevents detrimental effects on cell growth that can be caused by extractables released by film materials during bag manufacture and storage (**3**). Measured by a Chinese hamster ovary (CHO) cell–based cell-growth assay developed by Sartorius Stedim Biotech, the new film has demonstrated superior biological performance in accelerated aging studies and media-storage trials (**3**).

Similarly excellent results were recently published by the "Single-Use Technology in Biopharmaceutical Manufacturing" temporary working group (**4**). In their study, nine different cell lines and related media were used

Figure 1: Seed train of the 1,000-L fed-batch cell culture run starts with one cryogenic vial before six consecutive cell-expansion steps using single-use shaker flasks and bioreactors. A 17-day fed-batch production process followed in a BIOSTAT STR 1000 system. The entire duration from cryogenic vial to 1,000-L harvest on day 17 took 35 days.

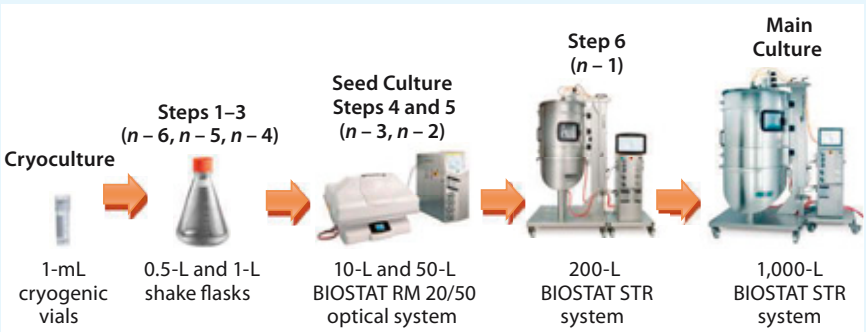


Table 1: Cultivation conditions of the 1,000-L production process comprising set points for pH, pO_2, and temperature and including ranges of gas flow rates and stirrer speeds as part of the pO_2 control cascade

Parameter	Set Point/ Range
Temperature	36.8 °C
pH	7.15
pO_2	60%
Stirrer speed	65–85 rpm
N_2	0–20 Lpm
Air flow	0–15 Lpm
O_2	0–60 Lpm

to evaluate different single-use bioprocessing bags from different vendors with regard to their ability to support cell growth.

In addition to biocompatibility, the robustness of Flexsafe bags has been confirmed through a number of different test methods, including an in-house water-burst test and worst-case application trials (**2, 5**). Finally, functionality and consistent, reproducible performance of these Flexsafe bags has been qualified following industry accepted methods (**2**).

In the study reported below, we discuss the performance of Flexsafe STR bags in a high–cell-density fed-batch culture of recombinant CHO cells producing a monoclonal antibody (MAb). Fed-batch cultures ran in 50-L and 1,000-L BIOSTAT STR single-use, stirred-tank bioreactors for 17 days, with the results compared with those obtained using a 5-L BIOSTAT B stirred-tank glass bioreactor. This fed-batch process is considered to be a suitable model system for process verification of Flexsafe STR bags regarding cell growth, productivity, and robustness in typical customer cell culture applications because such processes using CHO cells are widely used in the biopharmaceutical industry (**7**). The recombinant CHO cell line we used is the same one involved in the cell-growth assay our company established to support development of the new polyethylene film (**8**).

This fed-batch process is considered to be a suitable MODEL system for process verification of Flexsafe STR bags regarding cell growth, productivity, and robustness in typical biopharmaceutical applications.

Process Verification

We applied a recombinant CHO DG44 DHFR– cell line from Cellca GmbH to produce an IgG1-type MAb in a high–cell-density fed-batch process. Final MAb titers >7 g/L are typically reached with this cell line. We fed cultures using the chemically defined ActiCHO cell culture media system developed by Cellca GmbH: ActiCHO SM for cell expansion and ActiCHO P for production, with ActiCHO Feed-A, ActiCHO Feed-B, and a 400-g/L D-glucose solution from Sigma Aldrich.

The seed train for the 1,000-L fed-batch production process consists of six consecutive cell-expansion steps (Figure 1) starting with cryogenic vials. The inoculum cell density of all seed train steps was 0.2×10^6 cells/mL. For the first three expansion steps, we used disposable shaker flasks ($n - 6$, $n - 5$, and $n - 4$) incubated in a CERTOMAT CTplus CO_2 incubation shaker. We further expanded the cells in 5-L ($n - 3$) and 25-L ($n - 2$) working volumes using a BIOSTAT RM rocking-motion bioreactor. The last seed expansion step — at 200 L in a BIOSTAT STR 200 ($n - 1$) system — generated the required inoculum volume of ~100 L for the large-scale run. All single-use bioreactors used in our study were equipped with Flexsafe bags.

The 1,000-L production process started with a three-day batch phase followed by a 14-day fed-batch phase. We inoculated a BIOSTAT STR 1000 production bioreactor at an initial cell density of 0.3×10^6 cells/mL. Table 1 lists the cultivation conditions set for the production bioreactor. We used

Figure 2: Viable cell density (VCD) and viability of high–cell-density fed-batch processes in a BIOSTAT B 5-L glass vessel and BIOSTAT STR 50 and BIOSTAT STR 1000 Flexsafe bags; 5-L data include a standard deviation of cell density and viability for a total of four runs.

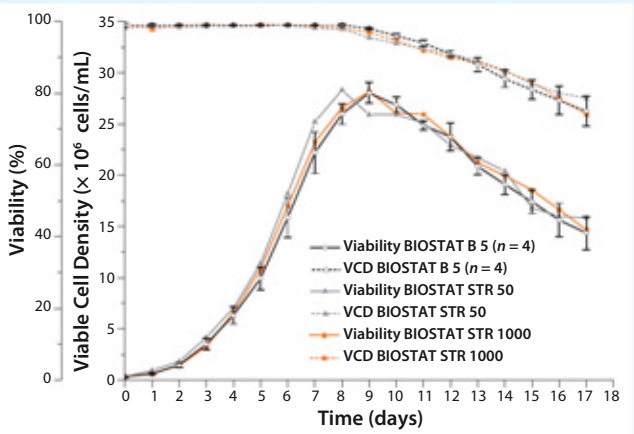


Figure 3: Monoclonal antibody (MAb) titer from high–cell-density fed-batch processes in a BIOSTAT B 5-L glass vessel and BIOSTAT STR 50 and BIOSTAT STR 1000 Flexsafe bags; 5-L data include a standard deviation of product titer for a total of four runs.

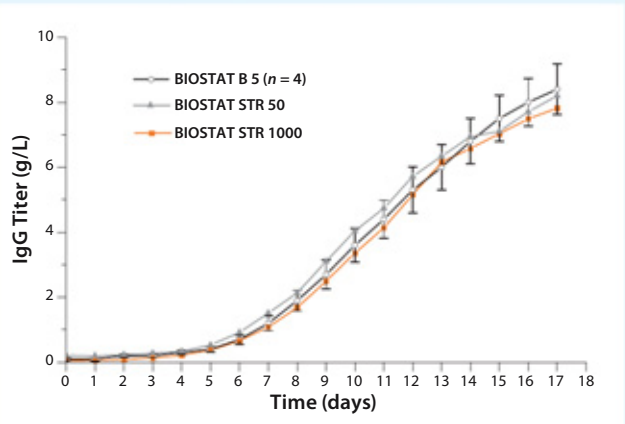
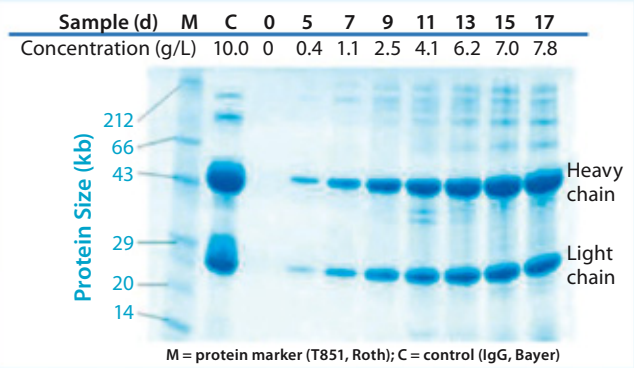
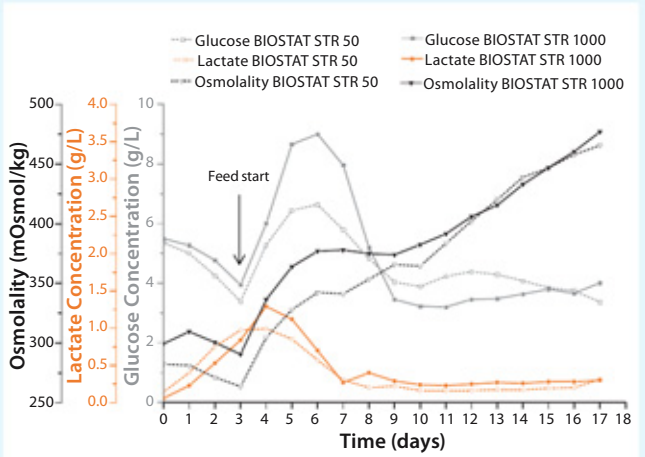


Figure 4: Quality of IgG1 produced during 17 days of high–cell-density fed-batch culture at 1,000-L scale assessd using sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS PAGE) under reduced conditions and Coomassie blue staining; in addition to samples from the process start and days 5, 7, 9, 11, 13, 15, and 17 post-inoculum, the graph shows a marker (T851 from Roth) and control (IgG from Bayer).



M = protein marker (T851, Roth); C = control (IgG, Bayer)

Figure 5: Glucose, lactate, and osmolality profiles of high–cell-density fed-batch processes in BIOSTAT STR 50 and BIOSTAT STR 1000 systems



two three-blade segment impellers and a ring sparger based on a previously performed characterization study on the bioreactor system, which suggests sufficient oxygen transfer using that set-up (**9**). As Table 1 shows, the system controlled pO_2 in a cascade-control approach involving stirrer speed and sparging with nitrogen (N_2), air, and oxygen (O_2). Carbon dioxide (CO_2) sparging controlled pH levels.

Superior Biological Performance at All Scales

The main aim of our study was to confirm the suitability and performance of Flexsafe bags for their final process application in both rocking-motion and stirred-tank formats up to the 1,000-L scale. Previous studies had already confirmed that the S80 polyethylene film material ensures superior and reproducible cell growth based on medium extraction trials using test bags (**3**). So our focus was to demonstrate not only that the film delivers excellent growth results, but also that the entire bag assembly performs well in a true and representative process application. During seed expansion ($n - 3$ to $n - 1$) using the BIOSTAT RM 10 ($n - 3$) and 50 ($n - 2$) as well as in the $n - 1$ BIOSTAT STR 200 seed bioreactor, we observed typical growth behavior that was comparable to what we have seen in cell expansions using shaker flasks. At the end of the three-day batch growth phase, viable cell densities (VCDs) of ~2 × 10^6 cells/mL with viabilities >99% were reached (data not shown).

Our focus was to demonstrate not only that the film delivers excellent growth results, but also that the ENTIRE bag assembly performs well in a true and representative process application.

Figure 2 compares cell growth and viability data of the high–cell-density fed-batch run at 1,000-L scale with data from 50-L scale using the single-use BIOSTAT STR bioreactor set-up. Our large-scale, single-use, bag-based bioreactors achieved excellent comparability in both runs. Those results are further supported by excellent comparability seen with small-scale reference data generated during four 5-L cultures in BIOSTAT B glass vessels. At 5-L, 50-L, and 1,000-L scales, all cultures achieved peak cell densities of ~28 × 10^6 cells/mL at >97% viability (measured by a Cedex HiRes cell-counting system from Roche Diagnostics) on day 9 post inoculum. Growth and viability profiles follow each other very closely at all scales. Notably, cell growth data from the BIOSTAT STR 50 and 1000 were within the standard deviation of the data from 5-L reference runs using conventional glass stirred-tank vessels. Hence, we observed no cell-growth–inhibiting effects with Flexsafe bags in the large-scale, stirred-tank format, which demonstrated excellent comparability and scalability of the bioreactor systems.

Accelerated aging studies of sample bags showed no impact on biological performance of the S80 film formulation during an investigated 36-month shelf-life period (**3**). Our study involved Flexsafe RM and STR bags that had been stored between one and 12 months. The consistent and superior cell growth obtained with the different scales and bioreactors further confirms those accelerated aging data using sample bags.

In addition to cell growth and viability, product formation (in this case an IgG1 MAb) is especially critical to establishing comparability of different cell culture systems. Figure 3 shows that, at all investigated scales, IgG1 titers were highly comparable and within the standard deviation of the 5-L glass vessel results. A final MAb titer of 7.8 g/L was achieved with the high–cell-density fed-batch

Figure 6: pO_2 profile obtained in a high–cell-density fed-batch process in a BIOSTAT STR 1000 system; in addition to pO_2 control performance, the graph shows stirrer rate, air flow, and nitrogen (N_2) and pure oxygen (O_2) gas flow rates (cascade control strategy).

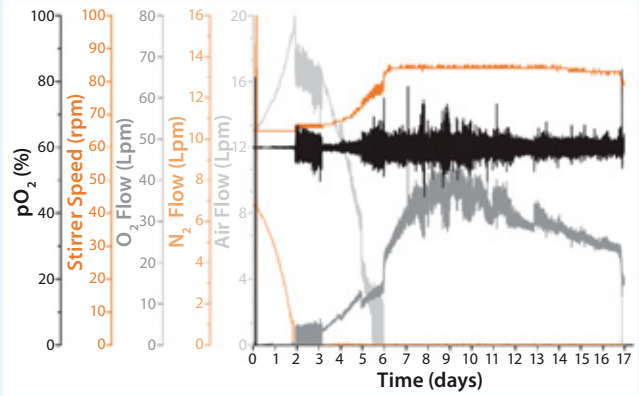
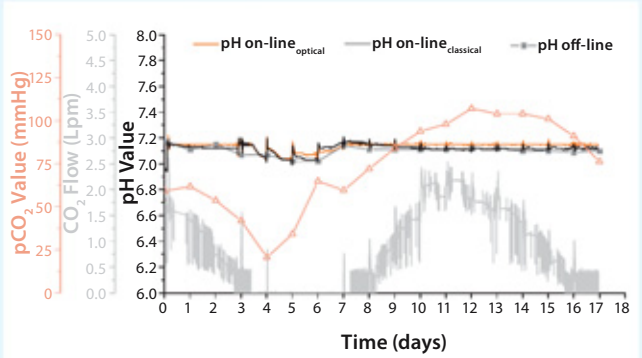


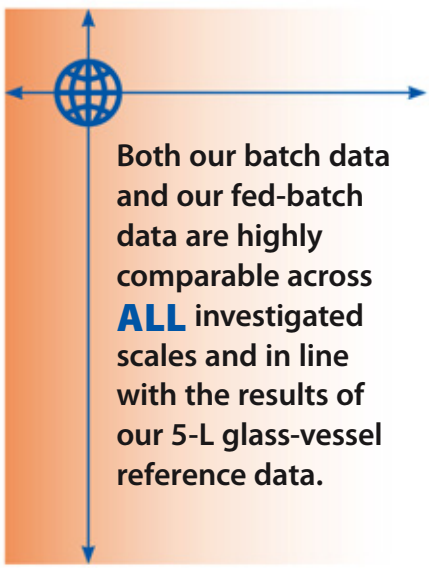
Figure 7: Comparing pH profiles of single-use optochemical probe (pH on-line optical) and conventional glass pH probe (pH on-line classical) with off-line pH measurement (pH off-line) for a 1,000-L high–cell-density fed-batch culture in the BIOSTAT STR 1000 system; further, CO_2 gas flow rates and off-line values of pCO_2



run at 1,000 L, which is comparable to 8.2 g/L achieved at the 50-L scale. Specific production rates ranged from 11 ng/cell on day 1 to 45 ng/cell on day 13. We used reduced sodium-dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to analyze product purity and potential degradation at different culture days of the 1,000-L high–cell-density fed-batch run and compared the results with an IgG1 control (Figure 4). Product-specific bands found at ~25 kDa and 50 kDa showed consistency with the control. Moreover, we discovered no significant impurities. Based on those data, we excluded the possibility of detrimental effects on productivity with Flexsafe bags. Negative impacts on product purity also seem unlikely.

Furthermore, we monitored glucose and lactate concentrations as well as osmolality during the fed-batch runs (Figure 5). Lactate concentration remained <2 g/L, below previously reported inhibitory levels (**10**). Glucose concentration was maintained at >3 g/L during the entire process. Osmolality ranged 300–470 mOsmol/kg and was kept below a previously established critical level of 550 mOsmol/kg (data not shown).

In essence, both our batch data and our fed-batch data are highly comparable across all investigated scales and in line with the results of our 5-L glass-vessel reference data. This demonstrates the superior biological performance of Flexsafe STR bags.



Successful Control of Critical Process Parameters

Minow et al. showed that detailed understanding of bioreactor performance enabled fast scale-up of a similar CHO fed-batch process (**11**). For our study, the extensive characterization of the BIOSTAT STR family with regard to oxygen transfer, mixing time, and power input summarized by de Wilde et al. (**9**) and the classical stirred-tank bioreactor design facilitated and simplified scale-up from 5 L to 1,000 L, as demonstrated by our highly comparable results.

We ensured successful pO_2 control at 60% ± 10% during the entire high–cell-density fed-batch process (Figure 6) using a multistage cascade-control approach based on pO_2 signals from a single-use optochemical probe. Adding antifoam and changing from air to oxygen flow control caused minor variations in the pO_2 value, especially at very low oxygen flow rates (Figure 6). We successfully controlled the pH value to 7.15 during the entire run (Figure 7) using signals from a single-use optochemical pH probe. During the fed-batch phase, daily supply of the highly alkaline Feed-B (pH 11) caused minor variations. For increased accuracy in pH control, we twice recalibrated the single-use probe signals (t = 3d, t = 6d) based on off-line measurements (ΔpH > 0.05 units). The single-use and conventional glass pH probes demonstrated excellent comparability during the 1,000-L run, which we confirmed with off-line pH measurements. This demonstrated the capability of single-use sensors for stable control of pH and pO_2 during a 17-day fed-batch production process.

We monitored pCO_2 values off-line using samples that were analyzed by an ABL800 Basic blood gas analyzer from Radiometer. Maximum values of 107 mmHg were reached on day 12 — well below a critical value of 150 mmHg reported by Zhu et al. (**12**). The pCO_2 values obtained during our 1,000-L high–cell-density fed-batch run were comparable to data obtained in the BIOSTAT STR 50 fed-batch run (data not shown). Thus, successful CO_2 stripping was achieved using the ring-sparger gassing strategy at the applied gas flow rates.

Conclusion

Our study successfully demonstrates the new Flexsafe STR and RM bag family's suitability using a state of the art, high–cell-density, fed-batch MAb production process using CHO cells. We have confirmed data obtained from γ-irradiated sample bags during development of S80 polyethylene film (**3**) in the final bag assembly with a typical cell-culture application growing recombinant CHO cells in chemically defined, protein-free cell culture media. In light of recently published *Dechema* results (**6**), it becomes obvious how important optimizing polyethylene films for cell-culture applications is to ensuring reproducible growth performance. We hope to pave the way for full adoption of large-scale, single-use bioreactors in late-phase and commercial manufacturing, in which quality, reproducibility, and assurance of material supplies are critical for safe and uninterrupted drug availability.

Direct scale-up from a 5-L conventional glass stirred-tank vessel (BIOSTAT B 5), through a 50-L stirred-tank single-use bioreactor (BIOSTAT STR 50) to the final 1,000-L single-use stirred-tank system (BIOSTAT STR 1000) verified again the unique scalability of our entire BIOSTAT family, which is based on classical stirred-tank principle and single-use materials with superior growth-promoting properties. These results are also in line with previous studies that showed good agreement of the engineering design space and key process parameters of BIOSTAT stirred-tank bioreactors for mammalian cell culture (**13**). In addition to achieving the same cell culture performance across all investigated scales, we also demonstrated efficient control of key process parameters such as pH and pO_2 in our very first high–cell-density fed-batch cultivation at 1,000-L scale.

In addition to similar design principles within the BIOSTAT stirred-tank bioreactor family, the combination of very effective CO_2 stripping with high oxygen mass transfer provided by a ring sparger enabled our direct and successful scale-up. Finally, the performance of the optochemical pH and pO_2 sensors demonstrates that a single-use bioreactor concept does not need to be compromised by insertion of classical probes that increase the risk of contamination. Our results convincingly show that a fully single-use cell culture process can be implemented easily and scaled reliably to production scale confirmed by excellent comparability of the 5-L, 50-L, and 1,000-L data.

Acknowledgments

Our thanks are due to the entire R&D upstream technology team for their technical contributions.

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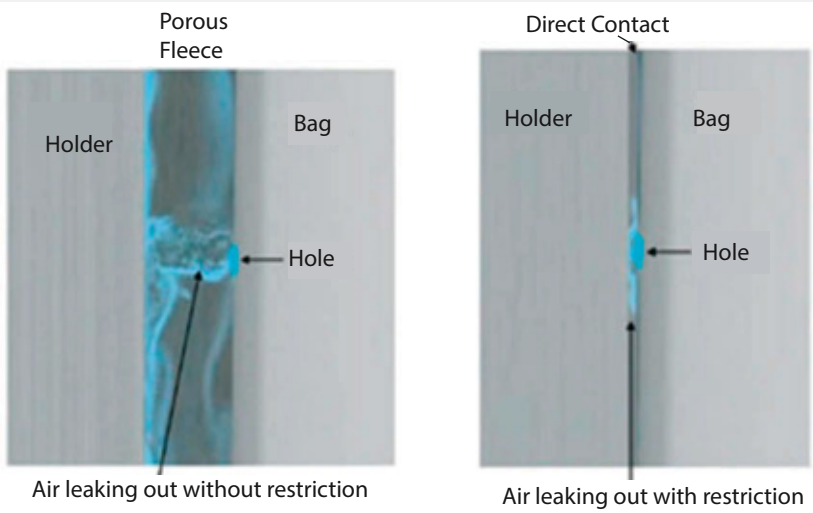
Pressure Decay Method for Postinstallation Single-Use Bioreactor Bag Testing

by Magnus Stering, Martin Dahlberg, Thorsten Adams, Davy De Wilde, and Christel Fenge

Single-use technology is well accepted today, and manufacturers' quality assurance programs ensure leak-free single-use bags upon delivery. But what about risks involved with installation and other handling errors? Operator training and implementation of suitable standard operating procedures (SOPs) are mandatory, but should they be the only ways to mitigate the risk of failures? In addition, more companies are advocating the use of ballroom concepts (**1**) for the manufacture of biopharmaceutical drug substances and drug products. However, how do you prove that applied unit operations are closed and intermediates are not subjected to risks of cross-contamination?

The high value of growth media and the duration of typical cell culture processes call for the highest degree of scrutiny when setting up such runs. A leaking bioreactor would generate a significant financial loss and jeopardize a carefully timed production schedule in a good manufacturing practice (GMP) production facility. In vaccine processes, it might also pose a risk to operator safety as well as the general environment and therefore must be mitigated under all circumstances.

Figure 1: Porous fleece prevents masking effect



Postinstallation Preuse Testing Helps Mitigate Risks

In terms of regulatory framework and feasibility, there is a clear difference between integrity testing sterilizing-grade filters and testing single-use bags. According to current European GMP regulations (**2**) — which are the most stringent among international guidelines — sterilizing-grade filters should be tested after sterilization both before use and immediately after use. Single-use bags have no such regulatory requirement. Nonetheless, a postinstallation preuse test of the entire single-use bioreactor system (including tubing) — capable of detecting typical leaks that might have been introduced by operator-handling errors — would greatly improve risk-mitigation capabilities in single-use production facilities. That could improve operator safety and financial security associated with maintained production capacity and on-time delivery. A typical example of a potential risk is the improper connection of different tubing elements during bioreactor setup, media preparation, and inoculum transfer. An operator error during such critical operations might result in a leak, potentially causing contamination at a later time and loss of a run.

Scanning Different Test Methodologies

More than 40 different test methods have been proposed for leak detection (e.g., according to EN1779 "Leak testing: Criteria for Method and Technique Selection") based on a number of technologies such as gas leak detection (integral or by probe), thermal imaging, flow measurement, and pressure decay and pressure increase.

Each technology has its own strengths and weaknesses in terms of

- accuracy and reproducibility
- rest time
- handling aspects
- condition of the bags after testing
- possibility of testing connections
- investment costs for equipment
- cost per test
- footprint of the test equipment
- contamination risks
- IP situation
- feasibility of in-place testing (point of use).

Those factors have to be carefully considered and balanced in developing a suitable bag-testing device for single-use bioreactors for GMP production of vaccines, monoclonal antibodies, and recombinant proteins.

Few Methods Qualify for Postinstallation Preuse Testing

There is a risk of potential handling errors when installing single-use bioreactors into their holders. So a reliable leak test for such bags must be performed at the point of use (preuse but postinstallation). Testing a single-use bioreactor bag in a separate device (as is usually done when applying gas leak detection) and then installing it into its bag holder would not permit detection of operator-handling errors. It would merely compensate for potential shipping incidences. Also, the complexity and costs associated with gas leak detection technology would be unreasonable to make it a routine testing method. However, proper packaging (by the manufacturer) and inspection of the cardboard box (by the customer) would detect shipping incidences.

Mass or volumetric flow measurement is a well-known technology, but it requires a very precise pressure control throughout the test. Slightly overshooting or undershooting the initial test pressure would directly affect the measured flow and result in false estimation of the test value. The bigger the volume the more difficult it is to accurately adjust the pressure. This methodology therefore would not be applicable to larger volumes.

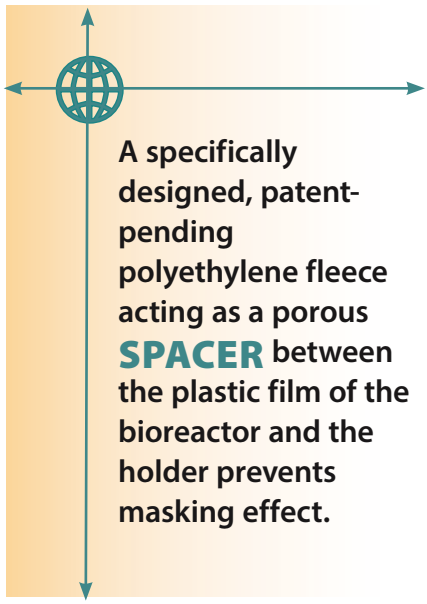
Pressure-decay technology, on the other hand, is a robust, easy to implement, cost-effective, and recognized technology that can be used to test a single-use bioreactor bag after installation in its final configuration directly in its holder. We have therefore used this approach to develop a reliable and predictive risk-mitigation tool.

Table 1: Minimum detectable leak sizes for different Biostat STR bag volumes

	50 L	200 L	500 L	1,000 L	2,000 L
Detectable minimum leak size at 20 min test time	50 µm	100 µm	200 µm	400 µm	TBD

Table 2: Test program parameters for different Biostat STR bag volumes

	50 L	200 L	500 L	1,000 L	2,000 L
Test pressure	50 mbar	50 mbar	50 mbar	50 mbar	50 mbar
Filling time	4 min	20 min	35 min	65 min	TBD
Stabilization time	20 min	20 min	20 min	20 min	TBD
Test time	20 min	20 min	20 min	20 min	TBD



Developing a Reliable and Reproducible Method

The objective of any test method must be to reliably identify potential damage of installed bioreactor bags covering bag seals, port welds, connections, and all bag surfaces. Any damage could result in a loss of bioreactor content or pose a biosafety risk.

We identified early on during the development of a method based on pressure decay that a leak in a plastic bag might be totally masked when pressed against a smooth, hard surface. Very little of the test gas can escape through the leak, and the reliability of the pressure-decay detection is nil. Therefore, performing pressure decay or flow measurement on a bag in a holder or even on a bag placed freely between two plates, without any porous spacer separating the bag from the flat surface, would not represent a reliable test set-up because of the masking effect.

Using a specifically designed, patent-pending polyethylene fleece acting as a porous spacer between the plastic film of the bioreactor and the holder prevents this masking effect (Figure 1). It delivers reliable and reproducible test values comparable to results obtained with the same test method on bag areas that are not covered by any hard surface.

Pressure-Decay Testing Methodology

The developed pressure-decay test method is based on the protocol from the American Society for Testing and Materials ASTM F2095-01 (3).The Sartocheck 4 plus Bag tester from Sartorius Stedim Biotech is used together with the above-described bag tester fleece that prevents masking of leaks that might have been potentially introduced during installation. It

Figure 2: Point-of-use testing of singe-use bags

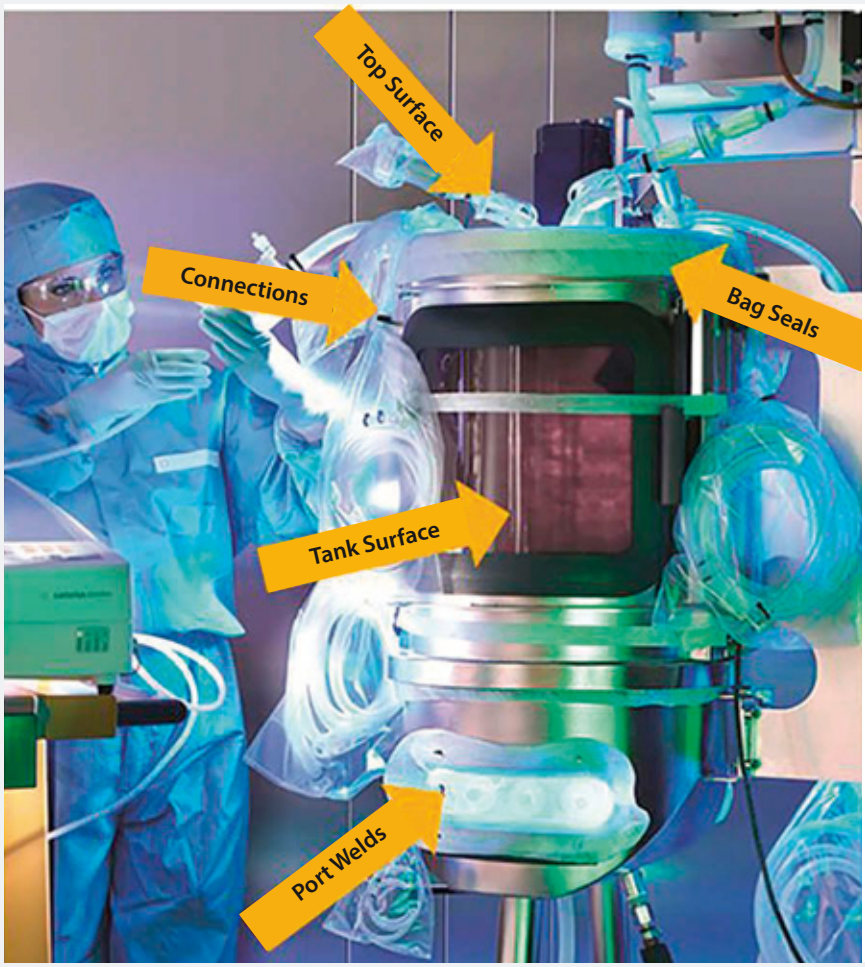


Figure 3: Engineering study results (BIOSTAT STR 200L bag)

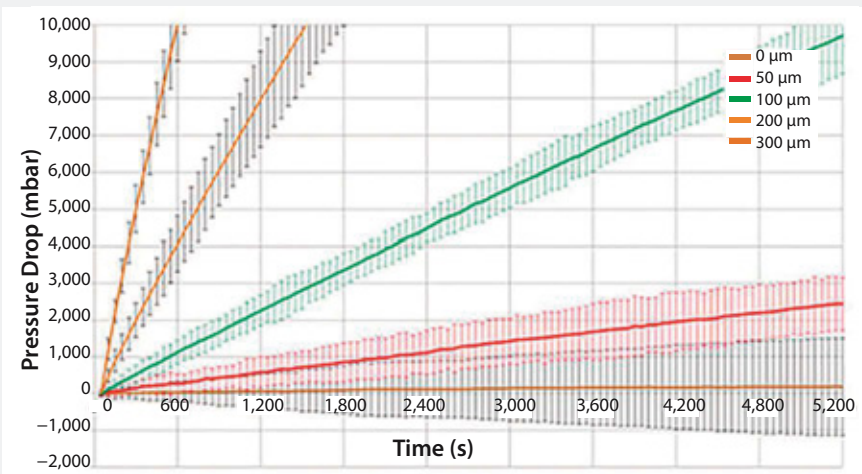


Table 3: Summary of maximum pressure decay for different bag volumes established during qualification study

	50 L	200 L	500 L	1,000 L
Test pressure	50 mbar	50 mbar	50 mbar	50 mbar
Maximum pressure decay during test time	1.2 mbar	1.5 mbar	2.5 mbar	3.2 mbar

ensures reliable point-of-use leak testing of single-use bioreactor bags postinstallation and preuse in its final bag holder.

Pressure-decay measurement is very sensitive to temperature changes. Therefore, the Sartocheck4 plus bag tester provides a temperature measurement of the environment and generates a warning message in case of excessive temperature drift during the test phase.

This pressure-decay leak test has been developed to reliably detect defects in BIOSTAT STR single-use bioreactor bags over the complete range from 50 L to 1,000 L. Qualification of the method at the 2,000 L scale is ongoing.

The bag is tested after installation, so potential leaks are detected in the bag walls, seals, or connections over the entire flexible bag system, including tubing. The test method is nondestructive and enables the implementation of a reliable and reproducible point-of-use test in bioproduction facilities (Figure 2).

The pressure decay during the test is measured and compared with acceptance criteria established during method qualification.

Qualification Limits Established During Initial Study

The validation approach consisted of two parts:

- an engineering study to establish the detection limit for different bag volumes
- formal qualification to verify the minimum detectable leak size and establish acceptance criteria of the test.

To determine the minimum detectable leak size, a simplified bag with only one bottom port connection for test gas application was prepared with multiple "defect patches" representing different defined leak sizes. A *defect patch* is a circular sheet of film with a laser-drilled and flow-calibrated leak diameter that was welded to the bag surface.

For reliable detection of a given leak size there must be a significant difference in pressure decay between a bag with the given leak size and a bag

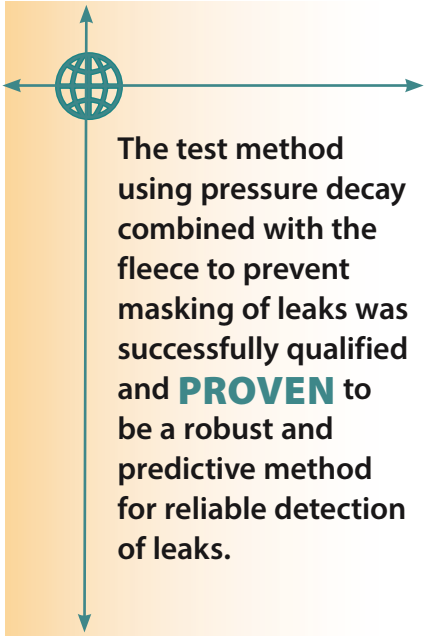
without defects. By repeated testing, the standard deviation of the test value was calculated for the various leak sizes and for bags without defects. The minimum detectable leak size was defined to be the leak size of which the lowest pressure decay value was at least six times higher than the standard deviation of the highest pressure decay value of a bag without leaks.

Figure 3 shows results for different defect patch sizes for a 200 L bag volume. The pressure decay measured for a 50 µm defect patch was overlapping with a bag without leaks. Therefore, a 50 µm leak cannot reliably be differentiated to a bag without leaks. On the contrary, a 100 µm leak size shows a clear differentiation to a bag without leaks.

In a second step, one standard bag without a defect patch and one standard bag with a defect patch of the previously determined minimum detectable leak size were used to establish the final test parameters. Table 1 shows the minimum detectable leak sizes for the different BIOSTAT STR bag volumes. Based on the results of the engineering study, the final test parameters for the qualification of the method were established (Table 2). Those parameters were used to generate a test program for each BIOSTAT STR bag volume using the Sartocheck 4 plus Bag tester.

Method Validation Confirms Reliable Detection of Leaks

During the final qualification study, the minimum detectable leak size for the different bag volumes was confirmed on a statistically significant number of standard bags from different routine production lots. Standard, nonmodified bags of different bag volumes and standard bags modified with a single defect patch representing the minimum detectable leak size were used. All bags were gamma irradiated. A minimum of 10 test repeats were performed per bag. Table 3 summarizes the maximum pressure decay measured for nondefect bags during the qualification study.



The test method using pressure decay combined with the fleece to prevent masking of leaks was successfully qualified and PROVEN to be a robust and predictive method for reliable detection of leaks.

All tested bags showed expected results: The nondefect standard bags passed the test, and the standard bags prepared with a single defect failed. Hence, the test method using pressure decay combined with the fleece to prevent masking of leaks was successfully qualified and proven to be a robust and predictive method for reliable detection of leaks during routine operation.

Fast and Robust Bag Testing Method for Routine Use

This new, qualified single-use bioreactor leak test based on pressure decay using a specifically designed porous fleece enables reliable and robust routine postinstallation and preuse testing. With this novel test method, the same level of risk mitigation and assurance of operating procedures can be achieved for single-use bioreactors as previously achievable only with conventional stainless steel bioreactors that could easily be pressure-tested before use.

The pressure-decay approach is easy to implement on a routine basis. The automated testing sequence and user guidance ensure a low risk of operator-handling errors. Monitoring the environmental temperature during the full test sequence further enhances reliability of the test results and prevents false conforming and false nonconforming test results. The equipment is both robust and cost-effective.

One saved batch can provide an instant return on investment. The leak test helps to prevent derailing of production schedule and project delays. Especially in processes where biosafety is critical (e.g., vaccine production), it is an indispensable tool for protecting operator safety.

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Total Solutions Support the Growth of a Dynamic Industry

A Conversation with Reinhard Vogt and Stefan Schlack

by Brian Caine and S. Anne Montgomery

While attending a conference at Sartorius Stedim Biotech in Göttingen, Germany, BPI publisher Brian Caine and editor in chief Anne Montgomery spoke with Reinhard Vogt (executive vice president of marketing sales and services, and member of the administrative board) and Stefan Schlack (senior vice president, marketing and product management). They discussed Sartorius's forward-thinking business strategies, its position as a total solution provider, and how the company's strategic goals mesh with its assessment of current industry directions.

Left to right: Stefan Schlack (senior vice president of marketing and product management, Sartorius Stedim Biotech), Brian Caine (publisher of *BioProcess International*), S. Anne Montgomery (editor in chief of *BioProcess International*), and Reinhardt Vogt (executive vice president of marketing, sales, and services, administrative board member of Sartorius Stedim Biotech).

Single-Use As an Enabling Technology

Caine: Your company positions itself as a total solution provider for the biopharmaceutical industry, with a special focus on single-use applications. Can you tell us what impact single-use has had on the market and what the future holds for the technology?

Vogt: For filter cartridges and capsules, single-use is quite an old concept. Today, though, single-use processes are much more complex and comprise part or indeed the whole production concept; they are replacing stainless steel containers and piping. However, it has taken a while for single-use to develop and for the solutions to become robust and scalable enough to operate at production level.

Assurance of Supply

Caine: Single-use technologies have reopened once-stalled markets, such as vaccines. As a total solution provider, how do you work with clients to assure them that Sartorius Stedim Biotech is the company that can take them from start to finish in their process?

Vogt: Single-use technologies have reached a quality level similar to stainless steel. Today, we offer a comprehensive technology and product portfolio. We source and monitor our raw materials, and we control the actual production of the single-use solutions as well. We bring our customers to our suppliers to ensure them that our suppliers' quality-management systems match our quality system and that they can rely on it.

Caine: What about other applications for single-use technologies and other indications? Do you see applications for your technologies in cell therapy, for example?

Vogt: We're watching the market carefully and have made some recent investments. We believe that we have an excellent portfolio already supporting this market. For example, our wave-style bioreactors are already used in this area, and very promising data have been published recently for our single-use, stirred-tank bioreactors. We're also cooperating with Lonza, which has a lot of experience in this area with its contract manufacturing services.

Montgomery: Where are we regarding the reality of completely single-use facilities?

Schlack: Actually, this is already reality for quite a few of our customers. In this respect, one of our

customers just received the ISPE "Facility of the Year" award for a completely single-use MAb process.We actually delivered the unit operations for upstream and downstream processing.

And we expect to see further significant activity especially driven by the so-called emerging economies. We believe that our industry faces four key challenges: capacity adaptation, smart process transfers, risk mitigation, and cost savings. For example, the Chinese biopharmaceutical market grows on average by 30% per year; some products grow by 70% or more. Fast capacity adaption will be a key success factor to meet the drug demand of the growing market. On one hand, it is important for our customers to avoid sunk costs due to underused capacity. On the other hand, they need sufficient capacity to secure market share. Especially for biosimilars, I believe that flexibility will be crucial and will be provided by single-use technologies

Another example is process transfer. When you put the right and scalable single-use technologies in your process development, you will be able to accomplish process transfers in 12 months or less. By contrast, process transfers based on stainless steel can easily take twice as long or even longer, depending on whether you have to build a new facility or adapt an existing one. No biopharmaceutical company can afford that amount of time: you have to be first to market to take full advantage of your patent or, in the case of biosimilars, take your share of the market.

We have developed very useful enabling technologies for single-use drug manufacturing facilities. For example, our new Virosart HF system is gamma sterilizable. You avoid sanitization, and at the same time you get an excellent virus filtration performance comparable with other market leading technologies. Another example is our FlexAct UD single-use solution for ultra- and diafiltration. It saves you up to six or more hours of preparation. This is especially critical when you think about product change-overs. Our customers typically look for solutions that reduce change-over times. Our membrane adsorbers are yet another example. They are easy to operate and fully scalable, so you don't need to invest in installing large chromatography columns for your polishing steps.

Providing a Total Solution

Caine: In terms of single-use, how do you define being a total solution provider, and what are the main drivers and hurdles in that market?

Schlack: We just talked about the challenges of the pharmaceutical industry. Single-use is a solution to address them. However, to offer a true alternative to stainless steel, we have to move away from focusing on components and individual products and offer complete single-use process steps. Using our FlexAct UD as an example, we provide a mixing bag, a mixer, vent filters, a controller, pressure transmitters and pumps, crossflow cassettes, a holder and a recirculation bag, a storage container

To be a true **ALTERNATIVE** to stainless steel, we have to move away from focusing on components and individual products and offer complete single-use process steps.

and bag, as well as tubing. And all that has to be combined with application and process know-how to make it easy for our customers to use. Furthermore, our customers require assurance of supply and transparent change-control procedures for the single-use components of the process steps. Sartorius Stedim Biotech is capable of doing this. However, managing this complexity is certainly a challenge, and we are prepared to develop our company to adapt to it.

Vogt: A total solution means exactly that — a *total* solution — not just the supply of components and the availability of scalable products for each process step, but risk mitigation and logistics as well. Some customers are looking for upstream solutions, some are looking for downstream solutions, and others may want a total production solution, wherever they are in the world. Some want an entire single-use factory; others want a hybrid solution. Some want as much as

possible to be single-use, but they might already have a 5,000-L stainless steel bioreactor. Sometimes, no single-use technology is available for a particular application. Our "total solution provider" strategy means that we understand what our customers expect from us: single-use products and integrated single-use process solutions, and perhaps most importantly, a partnership to jointly achieve the goal in the most effective way.

Montgomery: Talking about some of your single-use products, can you tell us a little more about your new Flexsafe bag concept and the new S80 polyethylene film?

Schlack: Sartorius Stedim Biotech has developed a new polyethylene (PE) film and family of bioprocessing bags to meet the single-use manufacturing needs of the future. Unlike any other film on the market used to make bags for biomanufacturing, our new S80 PE film, which is used in Flexsafe bags, is the result of close collaborations between Sartorius Stedim Biotech and our polymer and film suppliers. With this unprecedented partnership, we have developed a completely new polyethylene film structure and achieved excellent and consistent cell growth, robustness and unprecedented assurance of supply. With these unique benefits, Flexsafe enables the implementation of single-use bioprocessing throughout the entire drug manufacturing process: from development to production, from upstream to downstream, including cell culture, storage, shipping, mixing, and freezing and filling applications.

For example, take the issue of cell-growth inhibition in single-use bags.

This is a major issue for today's biopharmaceutical companies. What's the cause? It is related to a cytotoxic degradation product derived from a commonly used antioxidant. To overcome this challenge, you need a deep understanding of polymer and material sciences in combination with cell biology. We have exactly accomplished this deep understanding in Flexsafe development.

Flexsafe ensures excellent and reproducible growth, even with the most sensitive production cell lines. Cell-growth reproducibility is guaranteed by controlling the resins and the additives specifications and by setting a design space for the film-extrusion parameters. Independent laboratories have demonstrated that Flexsafe bags are free of cytotoxic leachables. No known toxic degradation product of common additives (bDtBPP) is detectable in water-for-injection (WFI) extracts. Furthermore we've established a secure supply chain even down to the resin manufacturer, with long-term contracts and true partnerships. Business continuity is achieved with a robust contingency plan that includes backup resin crackers and film extruders, multiple bag manufacturing and sterilization sites and safety stocks of resins and film.

Caine: How does the BIOSTAT STR 2000 affect your portfolio, and how does it benefit your clients? Will bioreactors need to be developed above a 2,000-L capacity?

Vogt: With increasing titers, I don't think that single-use bioreactors will need to go beyond 2,000 L. In some cases, that volume may not go above 1,000 L. Vaccine producers, for example, don't need a 2,000-L fermentor.

Schlack: I can agree to that; 2,000 L will be the manufacturing scale of the future, especially considering recent developments regarding intensified fed-batch processes. But it's equally important that you have a complete range. No customer buys a 2,000-L bioreactor from Sartorius Stedim Biotech and then another from a different supplier. You have to offer a complete range (2–2,000 L) so you can ensure straightforward and fast scale-up and scale-down, process transfers, and troubleshooting.

We also incorporate the same single-use sensors in each single-use bioreactor for complete comparability. We've worked hard to provide a complete upstream solution, which is why we acquired TAP Biosystems. The

ambr15 multiparallel minibioreactor has already become the industry choice for cell line and medium development. The ambr250 system that we recently launched is based on the same design principles as conventional glass stirred-tank systems and our BIOSTAT STR single-use bioreactor. At the same time, it is a multiparallel process-development bioreactor system enabling effective and fast quality by design (QbD) studies to determine the operating ranges of critical process parameters and the design space of the production process.

Vogt: Imagine that you have a single-use process and a single-use fermentor, and you source tubing from one supplier, the filter from someone else, the controller from yet another company, and so on. And then, the components don't fit together. Small, start-up companies aren't in a position to manage multiple vendors and support FDA inspections based on this level of complexity. And large biopharmaceutical companies are looking for sourcing efficiencies as well.

Montgomery: Not to mention the challenges of monitoring the supply chain for each of those components.

Vogt: Yes, and we're also trying to educate our customers about that.

Schlack: In process development, aspects such as assurance of supply, change control, and pricing security are not really critical. But when our customers move toward commercial production based on single-use processes, they become critical. Your manufacturing costs could significantly increase if those elements aren't in place when you go into current good manufacturing practice (CGMP) production.

As Reinhard said, to reach this level of assurance of supply, standardization is needed. But standardization will be possible only when the biotech industry understands that is has to reconsider the R&D mindset of selecting bits and pieces from multiple different sources — such as tubings from vendor A, filters from vendor B, sensors from C and so on.

Vogt: If you don't standardize a process, you can't automate it. We need more standardization in single-use technologies to improve quality, reduce operator errors, and decrease costs. The bag is comparatively cheap, but putting all the components together is expensive.

To Partner or Acquire?

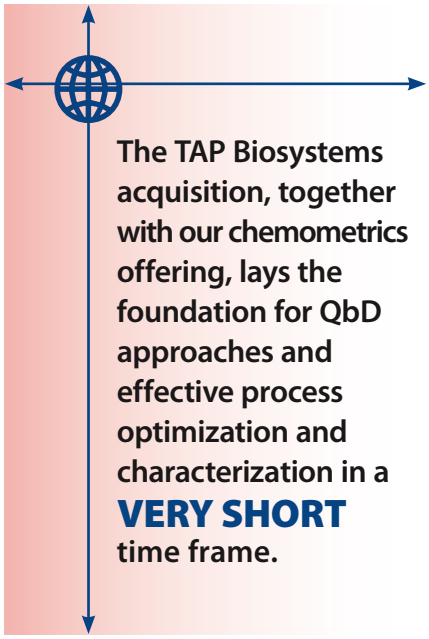
Caine: Having mentioned your association with SüdPack and the importance of collaborations, how do you decide whether to acquire or partner with a company? And how do you assess the value of one kind of relationship versus another?

Vogt: First, we determine what we consider to be a component rather than a key technology. Our key technologies are cell culture, membrane and single-use technologies. Once we decide on a key technology, we need to develop or acquire it so that we have R&D and the whole manufacturing under control and can ensure a sustainable supply chain. Sometimes, however, we identify a key technology but cannot buy the company for whatever reason, or we might be interested in only 10% of a company's portfolio. That's when we think about cooperation models. A good example is our agreement with Lonza. We wanted a media offering, and we also wanted to work with a company that had many years of experience in that area, with products on the market and a good brand name. Lonza uses 80% of its media for its contract manufacturing organization (CMO) activities, so it made no sense for us to buy the remaining 20%. Instead, we created a virtual joint venture without capital or legal components. Lonza does the production and oversees logistics; together we handle sales, marketing and R&D; and at the end, we share the profits.

Montgomery: Acquisition is clearly an important part of your business strategy. Can you offer more background on the purchase of TAP Biosystems?

Schlack: We believe that TAP Biosystems is the final piece in our upstream portfolio. When you do QbD studies, you have to do multiple small-scale trials. There's no better system available for that than the ambr250, which is truly scalable. We now can offer fully automated, multiparallel bioreactors, sensors and controllers, offering full scalability up to 2,000 L. So it's a really complete package. I'm very glad that it's now part of the Sartorius family.

Vogt: The acquisition became interesting when TAP Biosystems combined its automation expertise with single-use technology. TAP's know-how regarding stirred microreactors enables automated process optimization and the unparalleled development of cell lines for biopharmaceutical production by reproducing classical bioreactor conditions. The combination of automation and single-use is unique, and



multiparallel process development translates into enormous time and cost savings for customers.

The Future of Process Analytical Technology (PAT)

Caine: Let's switch to another important topic. During the conference, we've heard a lot about process analytical technology (PAT), QbD, and design of experiments (DoE). What do you think is the key to their successful adoption and commercialization?

Schlack: Our vision is to offer seamless transfer from process development to commercial production in upstream processing. We are very close to reaching this point. The TAP Biosystems acquisition, together with our chemometrics offering, was crucial in this respect because it lays the foundation for QbD approaches and effective process optimization and characterization in a very short time frame.

Caine: What's more important: technological advancement or additional education on existing technology?

Vogt: Both. Unlike the mature chemical industry, the bioprocess community is still on a learning curve. Our strategy has always been driven by finding out what our customers need, not what they want. That's a big difference. If you were asked 20 years ago whether you needed a smart phone, you might have said no. Today everybody uses them. That's a good example of need versus want.

Schlack: Timing is also important. It's often not about reinventing the wheel, but rather offering a complete solution. In our industry, you have technology adoption cycles of six to eight years or more, so you have to believe in your analysis of future trends — and keep on investing — even if you don't immediately get a good return on that investment.

Company Growth, Sustainability

Montgomery: Can you speak about planning for the future, how you recruit and train new staff?

Vogt: That's very important for us. We have a very good staff retention rate and good training programs. But I think it's also about company culture and giving young people a chance. You have to create a culture of appreciation and encouragement that goes beyond the framework of typical talent management.

Schlack: We need openness and room for people to gain experience. We have excellent people. As managers, we have to ensure that they can share their knowledge and exchange ideas. Of course, it helps to have a dynamic company in a vibrant market. We send people all over the world, which encourages them to develop their skills and expertise and keeps them loyal to the company. To lose people is to lose potential.

The Take-Home Message

Caine: Given that your market-facing statement for Sartorius Stedim Biotech is to be a total solution provider, what message are you trying to send to the market?

Vogt: First, customers should know that if they want to incorporate single-use processes, they should come to us! We have invented it, we are the market leader. And, of course, we go beyond components, technologies and applications into supply chain management. So customers can review our assurance of supply and business continuity plans. The other key message is that we have added products to our portfolio because we want to be best in class. We want to be the best in cell culture, in downstream processing, in filtration, and so on.

Schlack: We want to be a partner for the biopharmaceutical industry. We offer solutions that address key industry needs and challenges. And we work hard to close the final remaining gaps.

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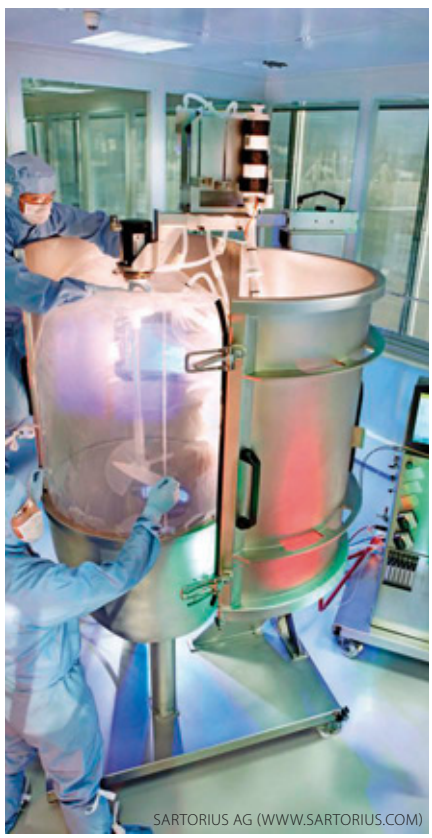
Disposables for Biomanufacturing

A User's Perspective

by Dr. Berthold Boedeker

The supply scenario for many biopharmaceutical drugs such as monoclonal antibodies (MAbs) is changing. With the implementation of personalized medicine resulting in drugs for specific, high-responder subsets of patients, market volume per drug will decrease. In addition, increasing fermentation titers of up to 10 g/L for MAbs are leading to smaller fermentation volumes necessary to accommodate individual biopharmaceutical market demands. That results in approaches such as flexible production in campaigns or decentralization of manufacturing using similar facilities with low risk of tech-transfer issues for regional markets.

In this regard, single-use technologies (disposables) play an important role in how biopharmaceutical development and production, particularly from mammalian cell culture, can nowadays be performed. Except for some large-scale unit operations such as centrifugation, chromatography skids, and UF/DF operations, all process steps can be performed in disposables up to a bag volume of around 3,000 L. Such steps include mixing, holding, and distribution of culture media and buffers; cell seed expansion; production fermentation; and cell removal by depth filtration, disposable chromatography columns, and UF/DF/virus filtration. Although many single-use units have been an integral part of biomanufacturing for a long time now through integration into hard-piped set-ups (filters, and so on), the real progress toward completely disposable processing came with development of single-use bioreactors (SUBs). Several systems are now available up to a fermentation scale of 2,000 L. However, there are still limitations with single-use technologies, particularly in the areas of pretesting and the quality of disposables, standardization and qualification of bags and connections, and validation of leachables and extractables, as well as dependency on individual solutions from different vendors.



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Advantages of Disposables

What are the major advantages of using disposables-based processing compared with standard production in a hard-piped, steel-tank–based setting? One major advantage is that presterilized single-use systems can be used in a laboratory-like environment. This is well suited for small-scale research and development activities, because no supporting engineering infrastructure regarding utilities, hard-piping, or automation (for example) is needed to set up and run such operations. This enables bioprocessing to be performed at a reasonable scale even in university laboratories. Another advantage is the time and cost savings in plant construction and operation. The main contributors here are lower capital costs; reduced consumption of utilities such as gas, electricity, and water (purified, WFI); no or limited hard-piping; and less-complex automation.

Time savings vary depending on the extent of disposables use. Most facilities still contain nondisposable unit operations. In hybrid designs, time savings in the early engineering project up to mechanical completion are sometimes marginal. But during start-up, including during

New processing approaches include use of disposables in DOE studies, feeding strategies, concentrated cell banking, and applications for **CONTINUOUS** processing.


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qualification and validation, time savings can be very pronounced (up to 70%) because equipment qualification using disposables is very limited, and no steam-in-place (SIP) and clean-in-place (CIP) processes are needed. Also the sometimes very lengthy cleaning validation of vessels and pipes that contact a product is not necessary for single-use because the bags are discarded after each run. Another advantage is the possibility to efficiently perform short product campaigns in multipurpose facilities, including fast product turnover by simply using new bags.

Addressing Limitations of Single-Use

On the other hand, a number of disadvantages, risks, and limitations of using disposables have to be addressed to make single-use-systems–based production a reliable alternative to standard production. First, there will always be a volume limit for handling and operating disposables. For fermentors and larger hold bags, that is expected to approach 3,000 L. For portable systems, the limit is currently in the range of 1,000 L. Another issue is standardization of single-use units and connections among vendors. Several integrated systems are being developed by individual suppliers, but those are not always compatible; that is, it is not possible to interconnect systems from different suppliers to a large, functionally closed processing unit. To comply with the desired second-supplier concept in biomanufacturing for SUBs, a company has to show biochemical comparability and consistent product quality in two bioreactor types before they can be used for commercial production. This is a substantial additional development and validation effort until two adequate systems are licensed. In addition, it is desired to get improved quality control by the suppliers. For example, bags should be pressure tested before delivery to reduce failure rates. It also would be advantageous to get full supporting validation packages, including extractables and leachables data as well as regulatory support files from the suppliers to make regulatory filing simpler.

Rapid Evolution of Improved Single-Use Technologies

Many new developments for continuously improving technical support, materials, and quality of disposables are shown in the articles of this supplement, illustrating that single-use technology is still a rapidly evolving area. Promising contributions are being made by vendors to assure end users that manufacturing can be performed reliably and with high quality as needed for pharmaceutical applications.

There are several contributions in this issue to addressing quality and reliability of bag supply. Among these are the use of new plastic surface films with fewer side effects on cell growth, improvements in film robustness, and supply-assurance strategies. Other contributions address quality-control issues such as point-of-use bag testing and SUB qualification strategies. Finally, new processing approaches are shown such as use of disposables in design-of-experiments (DOE) studies, feeding strategies, concentrated cell banking to avoid open cell handling, and applications of single-use systems for continuous processing.

All together, these new developments show that single-use technologies are maturing so that the desired situation may become reality: a fully disposable production facility with closed systems in a GMP-lab–like environment as an alternative or supplement to standard hard-piped, steel-tank based production. This would fulfill the desire for easy and fast plant construction, simple and reliable operation, high flexibility, fast product turnover, low cost of goods, and easy technology transfer to different regions.

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