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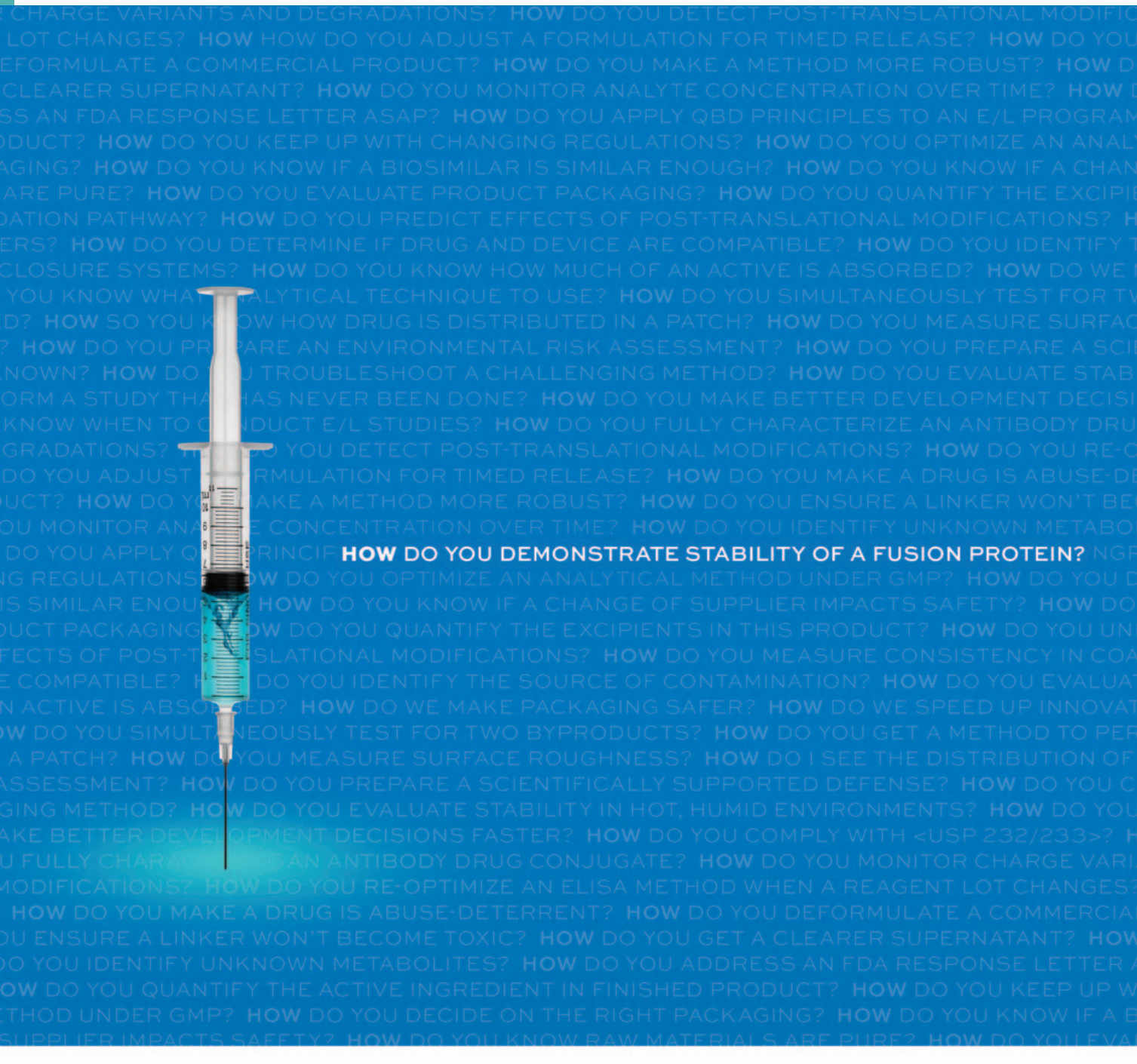
BioPharm INTERNATIONAL

August 2016

The Science & Business of Biopharmaceuticals



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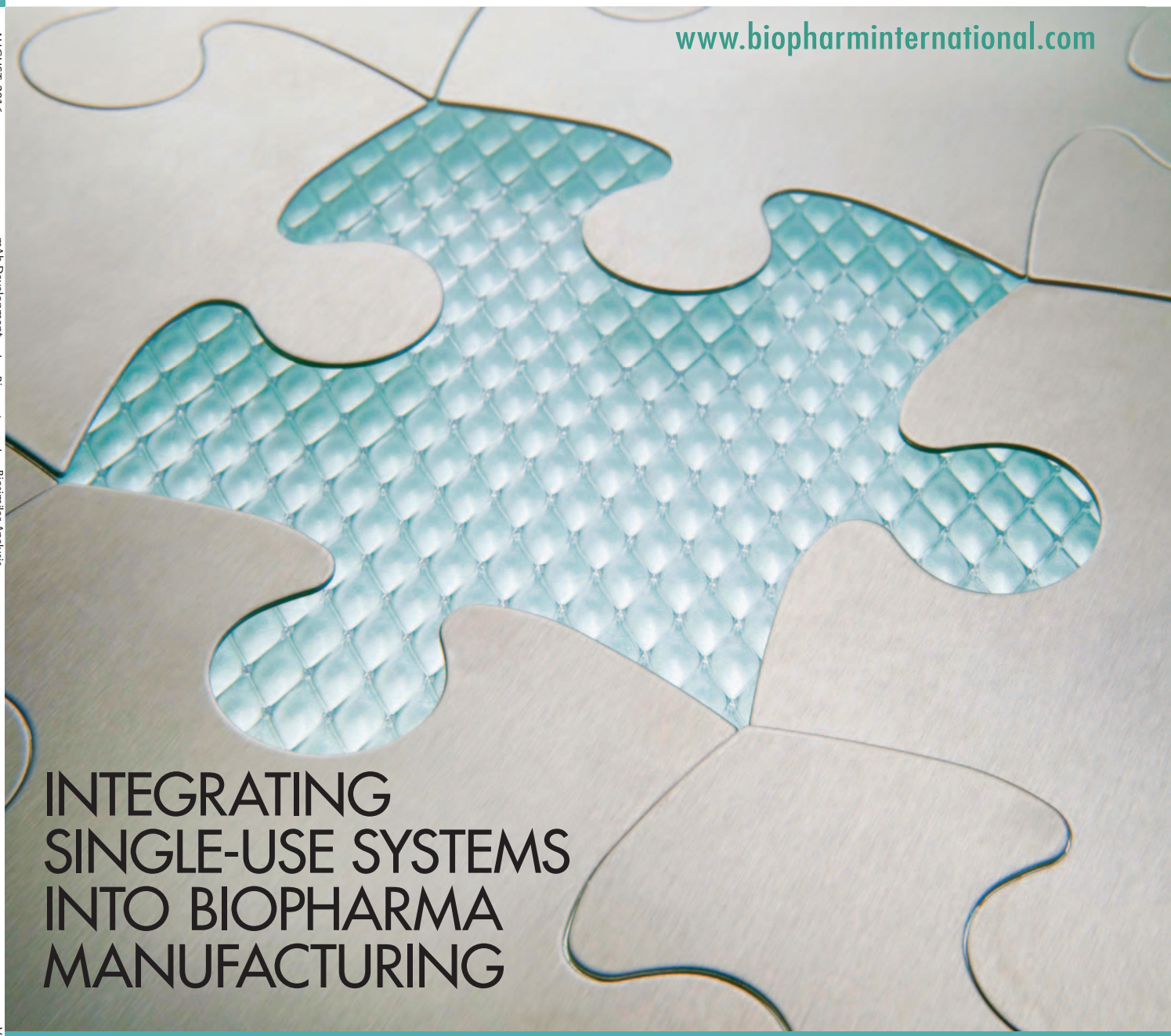

INTEGRATING SINGLE-USE SYSTEMS INTO BIOPHARMA MANUFACTURING

DOWNSTREAM PROCESSING
PROTEIN IMPURITIES POSE CHALLENGES

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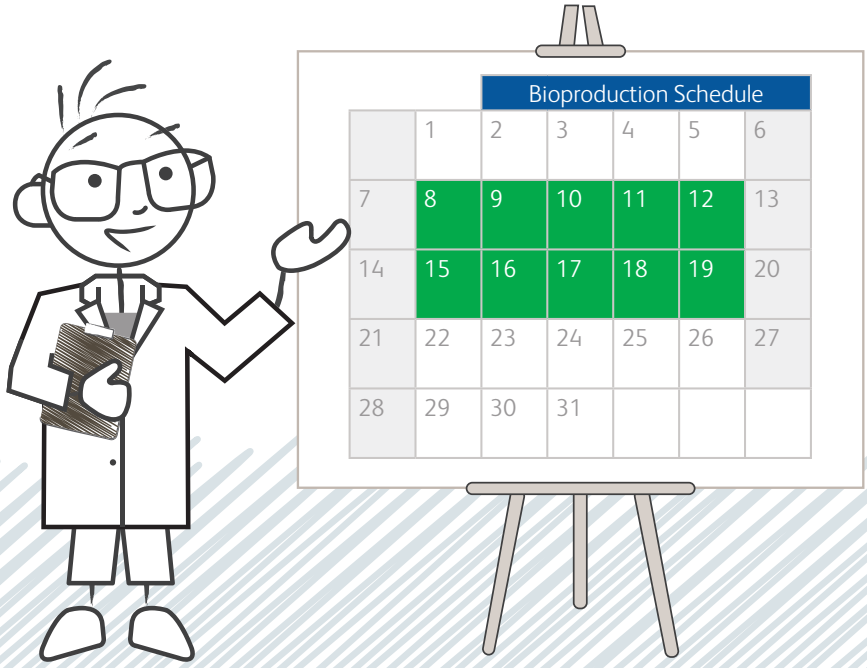

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Rita Peters is the editorial director of *BioPharm International*.

Congressional partisanship creates noise, but no funding for Zika virus research.

All Buzz, No Bite

The buzzing of mosquitos, a hallmark sound of summer, is now for some an ominous alarm. While a warning call that the Zika virus was coming to the United States was issued in the dead of winter, inaction by elected officials to reduce the impact of the virus has made the threatening buzz even louder.

As the thermometer rose, public health officials—particularly in the South—nervously monitored anticipated outbreaks of the virus. In mid-July, the Florida Department of Health announced an investigation into the first possible non-travel related case of the Zika virus in Miami-Dade County. Prior to this suspected case, more than 1300 cases of Zika were reported in the continental United States; however, all were related to travel to affected areas outside the US.

According to the Centers for Disease Control (CDC), Zika is transmitted to people through the bite of an infected Aedes mosquito, which can also spread dengue and chikungunya viruses. The virus can also be spread through sexual contact, blood transfusions, and laboratory exposure.

The CDC reports that the Aedes mosquito is present in 40 states and the District of Columbia. The method of transmission is simple: a mosquito bites an infected person, then carries the virus to the next person it bites. For most infected people, the symptoms of fever, rash, joint and muscle pain, headache, and conjunctivitis are usually mild.

For pregnant women and their unborn children, the threat is greater. Zika is known to cause microcephaly and other fetal brain defects; an infected pregnant woman can pass the virus on to her fetus during pregnancy. As of mid-July, more than 1600 serious birth defects were reported, mostly in Brazil.

The Zika virus is not new; it was first identified in 1947 in Uganda and spread around the world over the next six decades. In March 2015, Brazil reported its first outbreak and in October of that year reported an increase in the number of microcephaly cases among newborns. The number of cases grew, and on Feb. 1, 2016 the World Health Organization (WHO) declared a public health emergency because of clusters of microcephaly and other neurological disorders in some areas affected by Zika (1).

On Feb. 22, 2016, President Obama requested $1.9 billion in emergency funding for vaccine and diagnostic research, mosquito surveillance and control, public education programs, and improved health services for low-income pregnant women.

The response from Congress was unfortunately typical. Republicans argued that they did not want to provide a "blank check" for the administration to use for other purposes. The funding could be provided in next year's budget, they said, and argued for cuts in other areas to offset any emergency appropriations. Legislation offered by the Republicans, which would have provided less funding, contained provisions that were not acceptable to the Democrats. In the end, Congress went on summer break without providing any funding.

The Obama Administration reallocated nearly $600 million in funds from other projects to pay for Zika-related projects; state and local public health departments are also juggling budgets to increase mosquito control, education programs, and other prevention efforts.

New funding for vaccine research will have to wait. In the meantime, Congress will have to sleep tight, and hope the mosquitos don't bite.

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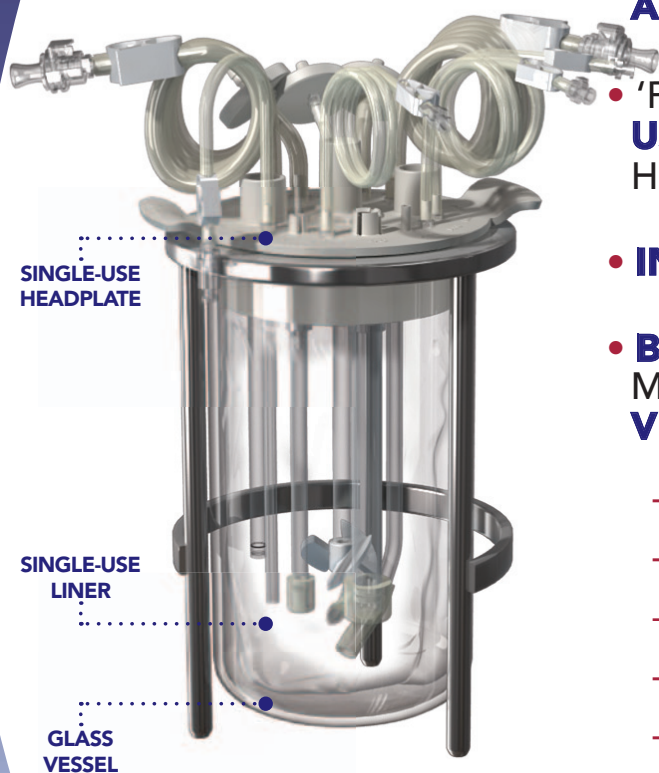

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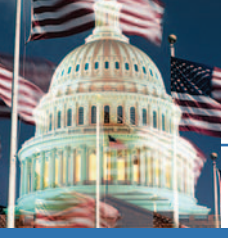

Quality Manufacturing Key to Stemming Drug Shortages

Regulators and manufacturers address economic and ethical issues for scarce medicines.

Drug shortages have lessened in the past two years, but remain sufficiently prevalent to raise serious concerns about ensuring patient access to crucial therapies. Ongoing shortages dropped from nearly 100 at the end of 2013 to approximately 60 a year ago, according to FDA's Center for Drug Evaluation and Research (CDER). The American Society of Health-System Pharmacists (ASHP), which compiles data from the University of Utah Drug Information Service, similarly reports a notable decline in new shortages over the past five years (1).

A main turning point was the enactment of legislation in 2012 that requires manufacturers to notify FDA in advance of likely supply problems for critical medicines. The legislation has helped the agency prevent and resolve such issues. The drug shortage situation "now is in a better place," commented CDER deputy director Douglas Throckmorton at the ISPE/FDA/PQRI quality manufacturing conference in June 2016. He credited expanded international cooperation and aggressive action by FDA's Drug Shortages Staff (DSS) in identifying alternative sources for scarce critical drugs. But supply problems still are "way too many," he said, noting that quality manufacturing failings remain the lead cause of supply disruptions, particularly with sterile injectables.

Jill Wechsler is *BioPharm International's* Washington editor, Chevy Chase, MD, 301.656.4634, jwechsler@advanstar.com.

PROBLEMS PERSIST

Shortages arise largely in markets where a small number of manufacturers—mostly generic-drug firms—serve the market, allowing little redundant capacity. To keep prices low, companies use common manufacturing lines for multiple products, and with 24/7 production schedules, there often is no time cushion for managing problems. At the same time, sterile products involve complex manufacturing processes, making it difficult to deal quickly with failures that can affect multiple products. And low profit margins on these operations discourage industry investment in updating or establishing new facilities.

As a result, clinicians and patients still struggle to cope with limited supplies of basic therapies, including cardiovascular drugs, electrolytes, and injectables used in emergency rooms and intensive care units. Vaccine shortages emerge regularly, the latest involving limited supplies of yellow fever vaccine in the face of a spreading epidemic in Africa. The World Health Organization (WHO) has recommended diluting doses to provide short-term but adequate protection to larger populations.

Shortages of drugs needed for acute care in emergency rooms and intensive care units are particularly serious, according to a study published in the May 2016 issue of *Health Affairs*. It found that more than half of nearly 2000 drug shortages reported between 2001 and 2014 to the University of Utah data system were for acute-care drugs where shortages pose a high risk of patient harm (1). The authors, from the Yale School of Medicine, report particular difficulties in finding alternative medications for scarce antibiotics, painkillers, sedatives, and saline intravenous solutions used daily in critical care. Substitutes, moreover, often are less effective, less safe, and more prone to medication errors during emergency care.

The analysts want FDA to do more to identify generic injectables produced by a single source and thus at risk of supply problems. They also support the use of tax credits, rebates, or temporary market exclusivity to encourage added generic-drug firms to produce needed injectables.

Similarly, the WHO executive board released a report in January 2016 on global shortages of critical medicines for children, with a focus on business and competitive factors that reduce access to low-cost injectables (2). WHO suggested that setting minimum prices or establishing multi-year global advance purchase commitments could help keep vital medicines on the market.

Broader interest in the economic drivers behind drug shortages has prompted the Pew Charitable Trusts and the International Society for Pharmaceutical Engineering (ISPE) to examine market issues affecting business continuity and supply-chain management decisions and investments related to drug shortages. More import of APIs and other components means that 68% of drugs in the United States contain ingredients from India and other counties, explained Frances Zipp, president of Lachmann Consultants, at the ISPE June conference. The project is interviewing key industry stakeholders on how factors such as market concentration, low margins, and product withdrawals affect shortage situations with an eye to presenting findings at the ISPE annual meeting in September.

Continued shortages of medically necessary medicines, particularly those used in emergency care and for treating cancer, also raise important ethical questions about allotting scarce supplies. This is particularly difficult in treating children with cancer, where care largely involves older generic injectables, explained Yoram Unguru, pediatric oncologist at Sinai Hospital in Baltimore, at the ISPE conference. A Working Group on Chemotherapy Drug Shortages in Pediatric Oncology issued recommendations for an ethical framework for allocating scarce life-saving drugs for treating childhood cancer in the February 2014 issue of the journal *Pediatrics* and examined the issue further in an article published online January 28, 2016 by the *Journal of the National Cancer Institute* (3, 4). Somewhat controversial is the group's advocacy for sharing drugs among institutions and its decision against favoring patients who participate in clinical trials.

Unguru urged pharma companies to do more to prevent shortages in the first place, and for all parties to adopt ethical allocation policies to maximize the benefit of scarce therapies. Oncologists and anesthesiologists are pressing for greater variety in vial sizes to reduce costs and help avoid drug waste when a full vial is not needed by a patient, a situation common in pediatric care.

EARLY WARNINGS HELP

Despite such ongoing drug supply issues, FDA's advance notification program appears to help prevent and mitigate shortage situations. Throckmorton reported that manufacturers are submitting approximately 200–300 reports of potential supply issues annually, which helped FDA avert 150 drug shortages in 2015. This strong response means that FDA has had to issue only two non-compliance letters so far to firms for failure to provide adequate warning.

After a manufacturer informs FDA of a likely shortage, CDER's DSS assesses the risk of disruption and helps devise mitigation strategies, working with CDER approval and quality offices and with the Center for Biologics Evaluation and Research (CBER) and FDA's Office of Regulatory Affairs (ORA). The analysts first determine if the product is medically necessary and checks for any applications under review that could help fill a gap or whether other manufacturers of the product can increase output.

CDER's Office of Process & Facilities (OPF) in the Office of Product Quality (OPQ) consults with DSS to assess alternative sources of supply and whether a facility with potential to provide a needed drug meets quality standards or requires a new inspection. Efforts by a manufacturer to increase production at an existing or new plant or to transfer technology to a new contract manufacturer may warrant accelerated review of microbiology, processes, and facilities, explained OPF acting director David Doleski at the ISPE conference. OPF may meet with firms at risk of shortage situations to discuss preventive strategies, coordinate a pre-approval inspection with ORA, and help resolve manufacturing issues.

FDA officials also can exercise enforcement discretion to facilitate access to new sources of crucial drugs. Import alerts may exempt medically necessary drugs and short supply situations. ISPE is developing a "gap tool" to help manufacturers assess how well they can avoid shortages by anticipating potential supply chain risks, forecast product demand, and provide redundancy in production operations. And industry commitment to quality manufacturing, said Throckmorton, is key to long-term prevention of drug shortages.

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Downstream Processing Continues to Worry CMOs

CMOs are working hard to improve performance by investigating new technologies for filtration and purification.

Downstream processing continues to be a significant pain point for contract manufacturing organizations (CMOs), and downstream-fueled bottlenecks don't appear to show any signs of abating, according to BioPlan Associates *13th Annual Report and Survey of Biopharmaceutical Manufacturing Capacity and Production* (1). In fact, nearly two-thirds (64%) of CMOs surveyed for the study report at least some bottleneck problems due to downstream processing.

DOWNSTREAM PROCESSING IMPACTING CMOs HARD

Downstream processing problems are not unique to CMOs, of course. It seems, however, that contract manufacturers are being hit harder than biotherapeutic developers, perhaps because of their working with multiple products, and their clients' demands for competitive performance.

More than one-quarter (27.4%) of CMOs surveyed said they are experiencing a serious bottleneck in capacity and overall production today due to downstream processing, a rate almost triple that of biotherapeutic developers (10.7%). And while almost 27% of biotherapeutic developers don't have any bottlenecks at the moment due to downstream processing, only a third as many CMOs (9.1%) can say the same.

Eric Langer is president of BioPlan Associates, tel. 301.921.5979, elanger@bioplanassociates.com.

This is the third consecutive year in which CMOs have reported a considerably greater degree of downstream problems than biotherapeutic developers, and that could point to more systemic issues than simply the nature of the CMO business. CMOs are unlikely to take on business for which they don't have adequate resources, so their continued troubles in this area could spell trouble for biotherapeutic developers. In fact, since the notable increase in capacity problems for CMOs due to downstream processing in 2014, biotherapeutic developers' own problems have edged up, from 42.6% reporting "serious" or "some" capacity problems in 2014 to 48% in 2016 (see **Figure 1**).

CHROMATOGRAPHY AND ULTRAFILTRATION STEPS

When it comes to specific steps that contribute to downstream-related capacity constraints, chromatography columns continue to be the main culprit. This year, 15.1% of all biomanufacturers surveyed reported "significant" or "severe" constraints arising from chromatography columns. On a more encouraging note, this figure has generally trended downwards in recent years (from 20.2% in 2008). Perhaps as a result, the industry's demand for new chromatography products appears to have slightly abated. This year, chromatography products ranked 6 out of 23 new product development areas of interest measured, after sharing the top ranking last year out of 22 areas tracked. Not too surprisingly given the larger constraints they face, CMOs (36.8%) were more likely than biotherapeutic developers (27.5%) to put chromatography products among their top five areas of new product development interest.

Despite the downward trend in significant problems related to chromatography, chromatography columns remain a bigger headache than other steps. One such step is depth filtration, which has also declined in its own right as a contributor to capacity constraints. This year, 8% of respondents reported at least "significant" constraints due to depth filtration, down from 12.7% in 2008.

While chromatography columns and depth filtration are gradually declining in impact, ultrafiltration steps are trending up as a prob-

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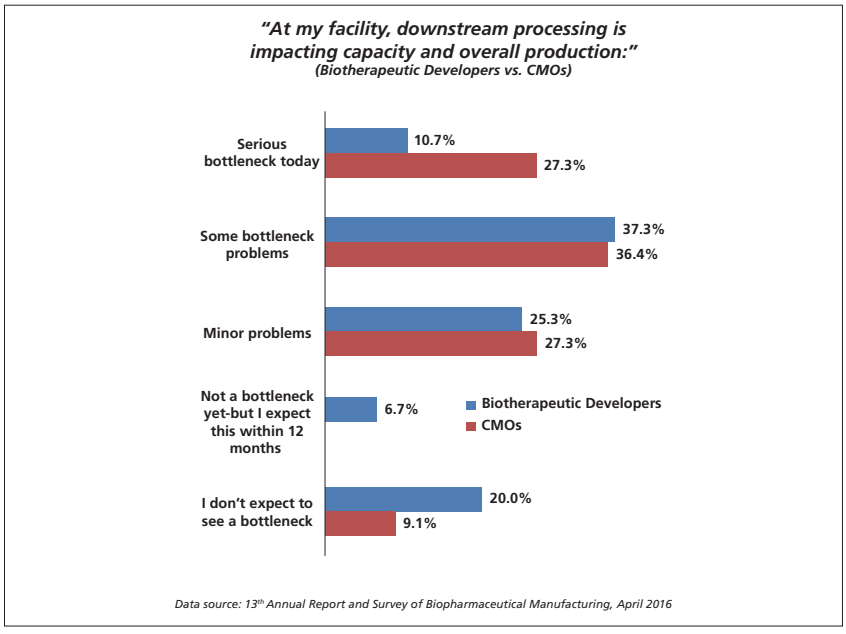
Figure 1: Impact of downstream processing on overall capacity: Biotherapeutic developers vs. contract manufacturing organizations (CMOs).



lem area. Ultrafiltration steps have led to "significant" or "severe" constraints for 9.4% of biomanufacturers, down slightly from last year's high of 10.7% but nevertheless well above the 6.5% registered in 2008. In fact, the past two years have been the first in which more "significant" or "severe" problems have been reported from ultrafiltration steps than from depth filtration.

SINGLE-USE DEVICES TO THE RESCUE?

To get a better sense of how the biopharmaceutical manufacturing market is positioning itself to deal with these problems, respondents were asked to identify actions they had implemented to improve downstream purification operations.

Among all survey respondents, the most common action taken was to cycle columns more frequently (50%). This was followed by:

- Optimizing running conditions (46.1%)
- Using or evaluating membrane-based filtration technologies (40.8%)
- Using or evaluating alternative ion-exchange technologies (39.5%)
- Investigating single-use, disposable downstream technologies (38.2%).

Fewer respondents this year reported taking multiple measures. This was particularly the case for developing downstream processes with fewer process steps (35.5%, down from 48.1% last year) and working with continuous chromatography purification such as simulated moving beds (13.2%, down from 22.2% in 2015).

At the same time, there is an increasing number of biomanufacturers actively identifying and assessing bottleneck points. The 32.9% reporting having done so this year is up from 22.4% in 2011, and marks the highest point so far. It appears that the biomanufacturing community is working harder to pinpoint the specific pain points to arrive at more targeted solutions.

Meanwhile a much larger proportion of respondents are investigating alternatives to protein A (27.6%) than actually switching to alternatives (6.6%), with this trend holding true for several years now.

Interestingly, two of the areas in which there were sizable declines in participation this year—investigating single-use products and developing processes with fewer steps—are CMO favorites. When respondents were segmented into two groups, CMOs and biotherapeutic developers, among CMOs, the most common ways of addressing downstream processing problems were to:

- Investigate single-use-disposable downstream technologies (66.7% of CMOs versus 34.3% of biotherapeutic developers)
- Develop downstream processes with fewer process steps (66.7% of CMOs, versus 31.3% of biotherapeutic developers).

Other actions taken by a majority of CMOs (each at a higher rate than biotherapeutic developers) were cycling more columns more frequently, using or evaluating membrane-based filtration technologies, and actively identifying/assessing bottlenecking points.

In general, CMOs appear to be implementing more actions than biotherapeutic developers to address their substantial downstream purification problems. In fact, the only six areas (of 19 identified) in which biotherapeutic developers show more enthusiasm than CMOs are:

- Using or evaluating alternative ion exchange technologies
- Process development to shorten cycle times
- Investigating alternatives to protein A
- Developing more efficient harvest/flocculation operations
- Investigating non-chromatographic separations (e.g., aqueous two-phase system [ATPS] , precipitation, crystallization, etc.)
- Implementing special handling such as freezing and precipitation.

CMOs' PASSION FOR NEW TECHNOLOGIES

CMOs have traditionally led the way in adoption of new technologies, and that continues to be the case in downstream purification technologies, too. CMOs and biotherapeutic developers were asked to identify the downstream purification technologies that they are considering this year. A majority of CMOs are considering:

- Use of high capacity resins
- In-line buffer dilution systems
- Single-use disposable tangential flow filtration (TFF) membranes
- Single use-prepacked columns.

Close to half of CMOs, meanwhile, are also considering using filters instead of resin chromatography.

By contrast, no technology attained even 40% interest among biotherapeutic developers, who are likely more wedded to current systems and more cautious about adopting new technologies. Developers reserved their greatest consideration for buffer dilution systems/skids (38.1%) and membrane technology (also 38.1%).

CONCLUSION

Data show that downstream purification continues to worry the biomanufacturing industry. Downstream problems, however, are contributing to more serious bottlenecks for CMOs than for biotherapeutic developers, with almost two-thirds of CMOs reporting at least a "significant" impact on capacity and overall production. This could be seen as a leading indicator of future problems for biotherapeutic developers, whose problems are gradually increasing in their own right.

CMOs are trying out various approaches to tackle these issues, often at a greater rate than biotherapeutic developers. Some of the biggest gaps between these groups relate to developing processes with fewer steps and to using single-use equipment in downstream processing. Indeed, a majority of CMOs are considering using single-use disposable TFF membranes and single use-prepacked columns. It remains to be seen the extent to which these will help. This much is clear: downstream continues to fail to keep up with the recent advances in upstream yield and titers.

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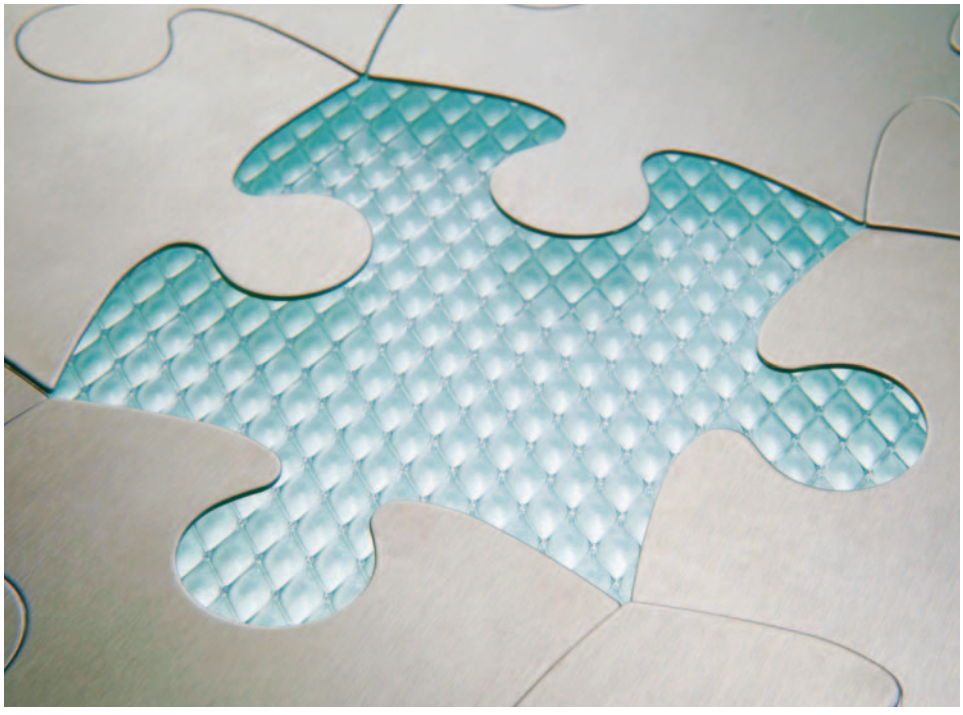
Integrating Single-Use Systems in Biopharma Manufacturing

Susan Haigney

Industry experts discuss the challenges of using single-use systems in biopharma manufacturing.



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To gain perspective on the use of single-use systems in biopharmaceutical manufacturing, *BioPharm International* spoke with Nandu Deorkar, PhD, vice-president of Research and Development at Avantor Performance Materials; Steve Miller, global head of Next Generation System Development, Life Science, Upstream and Systems Business Field Millipore S.A.S; Miriam Monge, director of process development and bioprocess platforms, Integrated Solutions at Sartorius Stedim Biotech; and Dr. Chris Chen, CEO of WuXi Biologics.

BENEFITS OF SINGLE-USE SYSTEMS

BioPharm: Which manufacturing processes benefit most from the use of single-use systems?

Deorkar (Avantor): Both upstream and downstream manufacturing processes benefit from single-use systems, particularly when manufacturers are producing buffer solutions or cell-culture media. Single-use systems reduce the time required to perform cleaning and cleaning validation. They also allow manufacturers to more easily—and quickly—turn over from one product to another, or from one batch to another batch. In addition, single-use systems can connect two unit operations, thereby minimizing hold time and enabling continuous processing. Finally, single-use systems are shown to reduce overall operating costs by minimizing or eliminating the need for clean in place (CIP)/sterilize in place (SIP), reducing analytical quality control costs—specifically for raw materials—and improving facility utilization time.

Miller (Millipore S.A.S): The main drivers for single use have altered little over the past 10 years. The processes benefiting most have issues around contamination and cleaning, reducing water consumption, ability to run multi-molecules in the same facility, and small-scale commercial production with low numbers of batches per year.

Monge (Sartorius Stedim Biotech): All those processes that require 1000 kgs per annum of product or less and can be manufactured in single-use bioreactors up to 2000-L scale or less can benefit enormously from working in single-use systems. Single-use [systems] offer increased flexibility to adapt to a wide variety of different processes at different scales coming through the pipeline. Single-use systems also enable increased productivity and reduced cost of goods. There are many end-user case studies out there to prove this is the case.

Antibodies, proteins, vaccines, cell therapy, and gene therapy all fall into categories of molecules that can benefit from being manufactured in fully single-use, end to end processes.

Chen (WuXi Biologics): Production scales between 15 L to 2000 L can all benefit from the use of single-use systems. Traditional fed-batch cell culture process as well as perfusion processes can be easily implemented in single-use systems. WuXi Biologics, an open-access R&D capability and technology platform company dedicated to biologics and a WuXi AppTec company, has embraced single-use technologies at full steam since 2010. The rapid adoption of single-use technologies has enabled us to quickly build up and expand our production capacities. As a contract development and manufacturing organization, single-use technology allows us to significantly reduce capital cost and facility construction time and increase running rate by quick turning-around between different products and eliminating cross-contamination risks.

ENSURING COMPATIBILITY

BioPharm: What measures should be taken when ensuring system compatibility between single-use systems?

Chen (WuXi Biologics): Different single-use products could have significant differences on design details, which might pose different degree of challenges when it comes to system compatibility between products from different suppliers. For example, agitation/mixing and gas sparging designs are quite different among the single-use bioreactors from the several major suppliers. The end-users need to carefully understand and evaluate the design features of the various products. The same challenges exist between single-use system and the stainless-steel systems. Process transfer using the actual production cell line and processes must be carried out to ensure system compatibility, with special attention on potential product quality impacts. Another challenge for a single-use bioreactor system is the lack of reliable scale-down model. Representative scale-down models of single-use bioreactors are typically at 50L scale, which is too expensive and also not practical/convenient to use.

Miller (Millipore S.A.S): Implementing single use brings with it new challenges that traditional facilities do not face, such as ensuring skids from different vendors have compatible connectors and common spare components such as clamps. Other, lesser-known challenges are related to packaging, installation and disposal, as each manufacturer may have different approaches, making operator life more complex and introducing opportunities for more errors.

Deorkar (Avantor): Single-use system components are typically made using flexible polymer films or plastic. Because of this, you need to make sure there are no extractables or leachables, which could impact cell growth, coming out of the system. These issues can impact product yield, quality, or stability. More importantly, you should evaluate the equivalency of the yield and the product quality coming out of the single-use system versus a stainless-steel system. If there is a difference between the cell growth in the stainless-steel versus single-use systems, you might see a difference in product, yield, and quality.

Monge (Sartorius Stedim Biotech): There are certain connectors that have become pretty standardized across the different suppliers, so connectivity between different single-use systems is not really an issue today. When talking about connectivity between single-use and stainless (in the case that a hybrid process design has been selected), autoclavable stainless-steel systems can be connected to single-use systems with a sterile connector, such as the Opta from Sartorius. The Opta is autoclavable and can be connected to a stainless-steel vessel and then autoclaved together with the vessel. This vessel can then be connected to a single-use system equipped with the Opta counterpart.

BioPharm: Have you seen any specific challenges in compatibility between single-use systems?

Monge (Sartorius Stedim Biotech): One of the challenges when testing single-use systems is that you need to compare the different testing methodologies used by the different suppliers. This is the case for extractables and leachables, pre-use and post-use integrity testing, and visible and non-visible particulates. It is not always easy to compare like with like.

Deorkar (Avantor): The long-term storage of solutions, such as buffers or cell-culture media, in single-use systems can generate extractables and leachables, which may cause impurities. One way to address this challenge is to prepare buffer or media on an as-needed basis by using solid materials that are customized for one single-use tank, rather than concentrated buffer solutions that are already in liquid form. We have provided customers with single-use powder materials in bags or other containers that are pre-weighed and compatible

with single-use systems. The packaging that contains the solid can be attached directly to a tank and the biopharmaceutical manufacturer can then make the solution, avoiding the long-term extractable/leachable compatibility issue.

MEDIA AND BUFFER SELECTION

BioPharm: What should be considered in regard to media and buffer selection when using single-use systems?

Monge (Sartorius Stedim Biotech): It is very important to review what tests are being carried out on the single-use systems and to which industry standards they comply. For example, at Sartorius we are aligning our testing methodologies with the industry standards that are identified as creating the greatest consensus in the industry both by suppliers and industry end-users and are recognized by the regulatory authorities. In this context, we are engaging actively notably with the American Society for Testing and Materials (ASTM) E55 working groups (E55: Manufacture of Pharmaceutical and Biopharmaceutical Products) developing standards for extractables and leachables, integrity testing at vendor factory and at end-user site, particulates at vendor factory, and at end-user site and cell growth.

When thinking about selection of single-use systems and films for media, the cell growth issue is of particular concern. Here we have proposed the development of a new standard assay assessing the suitability of specific plastics for cell growth applications in single-use. Recent industry reports demonstrate that current biocompatibility testing did not always detect cell growth issues. The discovery of a cytotoxic leachate raised industry awareness of the need for new evaluation techniques and testing to determine potential impacts of plastics used in single-use systems on cell culture plastics

Deorkar (Avantor): Before making a media or buffer selection, manufacturers should discuss packaging options with their materials suppliers. Often, the composition of packaging films in which a material is supplied is just as important as the material itself, because the packaging can impact the quality of the product.

Manufacturers should consider the impact that different buffer solutions or cell-culture media may have on the integrity of the single-use component—bags, for example—in which they are supplied. For example, some packaging for liquids supplied for single-use systems may not be 100% impermeable to air. This could change the buffer pH, conductivity, or stability of some solutions. By seeking out a supplier that can provide pre-weighed solid materials in packaging that is compatible with single-use equipment, manufacturers may be able to avoid this common issue. This type of packaging may also eliminate time-consuming material subdivision and kitting steps, which can reduce total cost of ownership, improve efficiency, and reduce the potential for contamination.

VALIDATION AND QUALIFICATION

BioPharm: What steps should be taken when validating suppliers?

Miller (Millipore S.A.S): There are many similarities to validating suppliers of traditional equipment: checking that the proposed equipment matches the process and is compliant with regulations and surface contact compatibility. What is different is that choosing single-use equipment is the beginning of a multi-year relationship between the customer and the supplier. Therefore, ensuring the supplier's commitment is more akin to a filter purchase than a system purchase. Assessing the supplier's process know-how, supply chain, manufacturing processes, support organization, and field force are much more important.

Another big difference for single-use versus traditional technologies is the importance of evaluating all scales of equipment, even if the procurement exercise is for the small scale only. A technology may work fine at a 50-L scale; however, when you scale to 2000-L processes, ergonomics and process robustness/optimization become a huge factor in the commercial operation. The difference with single use is that customers can be stuck with the technology they chose at the small scale and the conversion to alternative suppliers, even if there are significant advantages at the large scale, are too big a risk on the timeline to consider switching.

Monge (Sartorius Stedim Biotech): This is best verified through a supplier quality and supply chain audit. This includes reviewing the supplier quality system and manufacturing systems and ensuring that these are cGMP compliant. Ensure that the level of compliance is consistent across supplier sites, check the manufacturing environment in which the single-use systems are manufactured (ISO class 7 manufacturing environment for single-use systems is what the majority of end-users specify as a requirement), verify customer change notification process, and complaint management process.

Another important part of your single-use supplier audit includes supply chain security to ensure that the supplier has the systems in place to ensure delivery performance and offer best assurance of supply and business continuity.

BioPharm: What are the challenges of qualifying single-use equipment?

Miller (Millipore S.A.S): The challenges here are making the process robust. Qualifying the perfect scenario is easy, but anticipating damaged or dropped assemblies, qualifying how assemblies move from grey to clean space, and disposal are all exaggerated versus traditional facilities. These challenges can be minimized by ensuring equipment and assemblies come from the same supplier, as they should have qualification procedures as part of the total solution. ◆

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The Flexibility of Small-Scale Single-Use Bioreactor Solutions

Christian Müller, Lena Nielsen, and Torben P. Frandsen

The authors present a case study in which four single-use vessels were fitted to an existing bioreactor system.

Disposable technologies are being used more frequently for the development and manufacture of complex biologics in the biopharmaceutical industry (1, 2). Small-scale (bench-top) single-use bioreactors (SUBs) provide a faster turnaround time through the elimination of cleaning, assembling, and autoclaving operations. Small-scale bioreactors are the workhorses for process development and optimization, and because they are also scale-down models for process characterization, it is important that these vessels, whether reusable or single-use, replicate the design of production-scale bioreactors. In the present study, the authors examined four different presterilized single-use vessels (**Table I**) that were fitted to an existing bioreactor system with minor modifications, and also compared the performance with a control glass bioreactor.

The single-use bioreactors are configured relatively similarly; some had more options for sparging (i.e., micro- or macro-spargers) or for use of single-use (pH and DO) sensors. The vessel height/diameter and the impeller diameter/vessel diameter are fairly similar for these units; however, the system from BioBlu (Eppendorf) is relatively wider and has a larger impeller. The UniVessel (Sartorius) and the Mobius (Merck Millipore) vessels are only available in one size, while the BioBlu exists in several versions ranging from 65 mL up to 40 L including packed-bed versions and furthermore with models for microbial applications. The CerCell bioreactors can be user-designed (i.e., variations in diameters, height, impellers/turbines, connections, single-use-sensor brands) using an online tool. The vessels can be designed ranging from 250 mL to 30 L in polycarbonate and also for microbial and packed-bed applications.

FITTING THE SUB TO THE EXISTING BIOREACOR SYSTEM

For the bioreactor evaluation, an existing control system (i.e., a BIOSTAT B-DCU II system from Sartorius) was used. This system's probes for temperature, pH, and dissolved oxygen (DO) could be used in all systems directly. For the stirrer, motor adaptors were obtained from the different vendors. The bioreactor temperature could in some cases be controlled using electrical heating blankets. Because this required modifications of the circuitry to the control system, it was decided to control the bioreactor temperature using the existing Biostat B-DCU II water-based thermostat system instead. This was done by attaching the thermostat system via Rectus quick connect couplings to Flexijacket (by Service-Tekniker.dk), which is a flexible black silicone water heating jacket that can be customized for the bioreactor size needed. Flexijackets with a length of 44.4 cm could be used for all the single-use bioreactors, and the temperature could be precisely controlled in the vessels.

TESTING THE SINGLE-USE BIOREACTORS

In this study, platform fed-batch cultivations were conducted using a Chinese hamster ovary (CHO) expression platform (CMC Biologics' CHEF-1) (3, 4) as a model system with a cell line expressing a model fusion protein. For the experiments, the four disposable bioreactors and a control 5-L glass bioreactor were run side-by-side on two separate occasions using the process parameters described in **Table II**. In all vessels, open hole or ring spargers were used. The

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Table I. Parameters and characteristics of the evaluated bench-top single-use bioreactors (mammalian cell culture).

Bioreactor	UniVessel SU	BioBlu 5c	CellVessel	Mobius CellReady	5L Glass
Vendor	Sartorius	Eppendorf	CerCell	Merck Millipore	Sartorius
System	Disposable	Disposable	Disposable	Disposable	Glass (control)
Total volume	2.6 L	5.0 L	3.0 L*	3 L	6.5L
Max. working volume	2.0 L	3.75 L	2.25 L*	2.4 L	5.2 L
Min. working volume	1.0 L	1.3 L	N/A	1.0 L	2.5 L
Impeller type	3-blade pitched impeller, 30° angle	3-blade pitched impeller, 45° angle	3-blade marine impeller*	3-blade marine impeller	3-blade pitched impeller, 45° angle
Number of impellers	2	1	1*	1	1
Impeller diameter	54 mm	100 mm	60 mm*	76.2 mm	68 mm
Vessel diameter (inner)	130 mm at top (1.5° slope)	170 mm	130 mm*	137 mm	158 mm
Impeller diameter/ vessel diameter	0.42	0.59	0.46	0.56	0.43
Vessel height	240 mm	256 mm	225 mm*	249 mm	335 mm
Vessel height/ diameter (H/D or aspect ratio)	1.8	1.5	1.7	1.8	2.1
Power Number, Np	1.2	1.3	N/A	0.3	N/A
Thermowell pocket for pt 100 temperature sensor	Yes, 8 mm	Yes, but narrows towards end (< 7.6 mm)	Yes, 8 mm*	Yes, 7.6 mm	Yes, 8 mm
Sparger type (used in this experiment underlined)	L-sparger	Porous Microsparger or macrosparger	L-sparger*	Sintered polyethylene microsparger or open pipe	Ring sparger
Sparger hole diameter	14 x 0.5 mm	7-12 µm or 0.7 mm open hole	10 x 750 µm holes*	15-30 µm pores or 2.3 mm hole	14 x 0.5 mm
pH probe	Single-use PreSens or reusable	Single-use PreSens or reusable	Single-use or reusable*	Reusable	Reusable
DO probe	Single-use PreSens or reusable	Reusable (into sleeve with permeable gas membrane)	Single-use or reusable*	Reusable	Re-usable
Can stirrer adaptors be supplied?	Yes	Yes	Yes	Yes	N/A
Comments	Requires SU connection box for use of single-use sensors; Torospherical bottom design	A 'slim' version of the vessel, BioBlu 3c, with an aspect ratio of 2.0 and the same max. working volume is also available. Single-use sensor requires connection box.	*Can be user customized/defined		Glass vessel used as control

single-use bioreactors have slightly different total volumes, working volumes, and stirrers; therefore, the initial working volume, overlay airflow, and stirring rates were adjusted to fit each bioreactor and to stir with similar power input per unit volume (W/m^3) (see **Table II**).

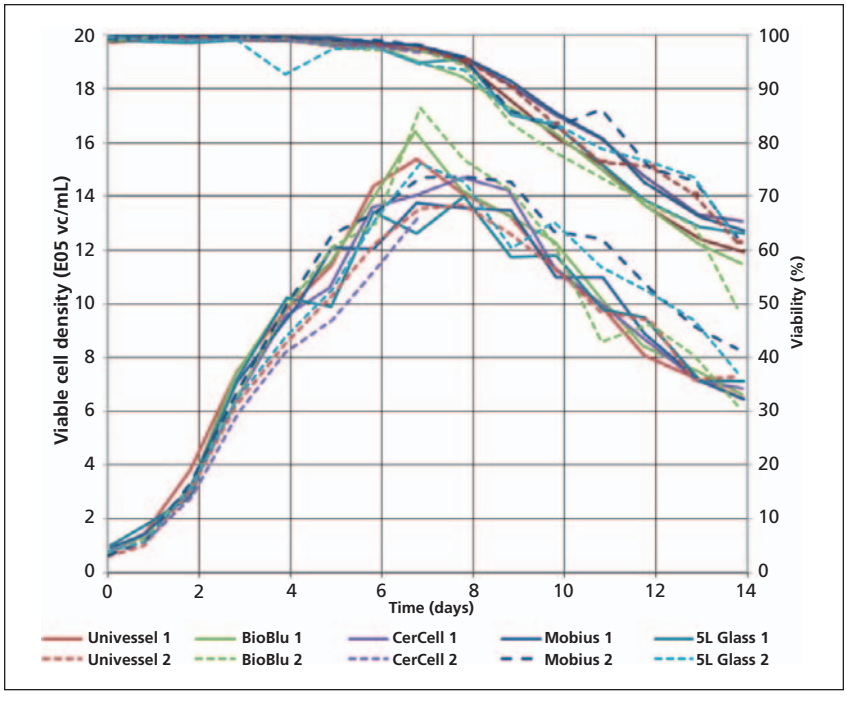
For investigation of the cell-culture performance, parameters including cell growth, viability, and metabolism and on-line trends for temperature, pH, and DO were compared. Samples were analyzed daily on a Cedex HiRes (Roche) and a Bioprofile 400 system (NOVA Biomedical), and after the cultivations were terminated, product concentration and key product quality characteristics were analyzed.

In each of the two experiments, fed-batch cultivations were conducted for 14 days in the four disposable bioreactors and the control glass bioreactor. As shown in **Figure 1**, viable cell density and cell viability were comparable during the course of the experiment for all bioreactors (single use and control) in both experiments.

Table II. Bioreactor process parameters.

Media			
Starting medium	CD-CIM1	Feed medium	Feeds A, B, and C
Set points, controls, and feeds			
Parameter	Set Point	Acid	CO_2 in overlay
Inoculation viable cell density	0.6E+06 viable cells/ml	Base	2M NaOH
Initial Temp.	37°C	Antifoam	ADCF Antifoam used if foam level is > 2 cm
First Temp. Shift	34°C from day 3	Second Temp. Shift	32°C from day 10
pH	6.90 (DB ±0.15)	Culture Duration	14 days
Dissolved oxygen	60% (optical probes)	O_2 on demand through the open hole/ring sparger is used	
Feed	Bolus feeds of 1.25%, 2% and 5% of initial bioreactor volume were given on days 3, 5, 7, 9 and 11		
Specific volumes, overlay, and stirring rates used			
Vessel	Initial working volume	Overlay airflow	Stirring rate
UniVessel SU (Sartorius)	1420 mL	90 mL/min	100 rpm
BioBlu (Eppendorf)	2660 mL	120 mL/min	80 rpm
CellVessel (CerCell)	1600 mL	90 mL/min	140 rpm
Mobius (Merck-Millipore)	1700 mL	90 mL/min	150 rpm
Glass vessel (Sartorius)	3680 mL	200 mL/min	150 rpm

Figure 1. Viable cell density and cell viability vs. cultivation time for the four disposable bioreactor runs compared to the control during 14 day fed-batch. Both Run 1 and Run 2 are depicted. Data were obtained daily from a Cedex HiRes (Roche).



Online trends of total CO_2 and oxygen usage, DO, temperature, and pH control for all bioreactors showed similar trends (data not shown). Comparable lactate, CO_2, and offline pH profiles were also obtained (**Figure 2**), indicating that the difference between the different single-use bioreactors and the glass bioreactor control is small, and that the cell-culture parameters are comparable even though the bioreactor set-ups are different.

Determination of the product titer (**Figure 3**) over the course of the two fed-batch experiments shows that the cells produce a comparable amount of product in the different bioreactor units. Furthermore, analysis of selected product characteristics (e.g., glycosylation, aggregation, and product-related variants) (data not shown) showed that the product is similar in the different bioreactors.

BIOREACTOR EASE-OF-USE EVALUATION

Generally, the use of single-use bioreactors is a major advantage for an efficient workflow during upstream process development and optimization because it can significantly reduce bioreactor down-time (or turnover time) by eliminating cleaning, assembling,

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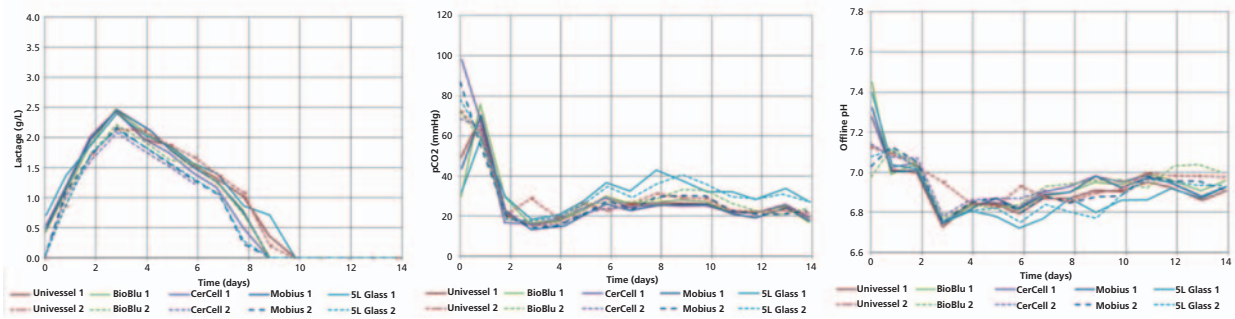
Figure 2. Lactate, pCO_2, and offline pH vs. cultivation time for the four disposable bioreactor runs compared to the control during 14 day fed-batch. Both Run 1 and Run 2 are depicted. Data were obtained using a Bioprofile 400 (Nova Medical) of offline pH-meter (Hamilton).



and autoclaving. Key operations such as pH and DO probe calibration and maintenance, media preparation, feed-tubing assembly, and sampling/analysis will still be needed—even if single-use pH or DO sensors are also implemented. The authors estimate, however, that use of these vessels can reduce the preparation time substantially.

During these experiments, a user evaluation was carried out examining the ease-of-use of the different single-use vessels for the CHEF-1 CHO platform process described. The amount of weldable connections were considered (3 feeds + glucose, antifoam, and base was in this case needed) as well as ease of sampling, how well condensate could be removed from the gas exhaust, amount of foaming, harvest options, and how well the stirrer adaptor worked.

The overall conclusion was that the four different single-use bioreactors tested all provided ample solutions for the examined fed-batch process. All bioreactors could be used as intended, and required little or no adjustment to be run effectively. Regarding feeding/sampling connections, all units provided weldable C-Flex connections (1/8 inch) for feed addition to the top of the bioreactor and some provided weldable C-Flex connections for addition to the bioreactor liquid. The BioBlu and UniVessel units also provided larger diameter (1/2 inch) weldable tube with Kleenpak Quick Connections, and the Mobius provided the large diameter (1/2 inch) weldable tubing connected to smaller weldable diameter (1/8 inch) tubing for flexibility. The CellVessel was user-designed with only the smaller 1/8-inch diameter weldable tubing due to the relative small volumes needed to feed/sample/harvest from the 2–5L bioreactors, and to minimize volume hold-up in the tubing. For sampling, aeration and agitation of the single-use bioreactors all worked similarly well despite the different solutions (e.g., regarding the stirrer adaptors). With respect to solutions for off-gas exhaust, the task of avoiding pressure

Figure 3. Product concentration vs. cultivation time for the four disposable bioreactor runs compared with the control during 14-day fed-batch. Both Run 1 and Run 2 are depicted. Data were obtained using a Protein A high-performance liquid chromatography (HPLC) method.

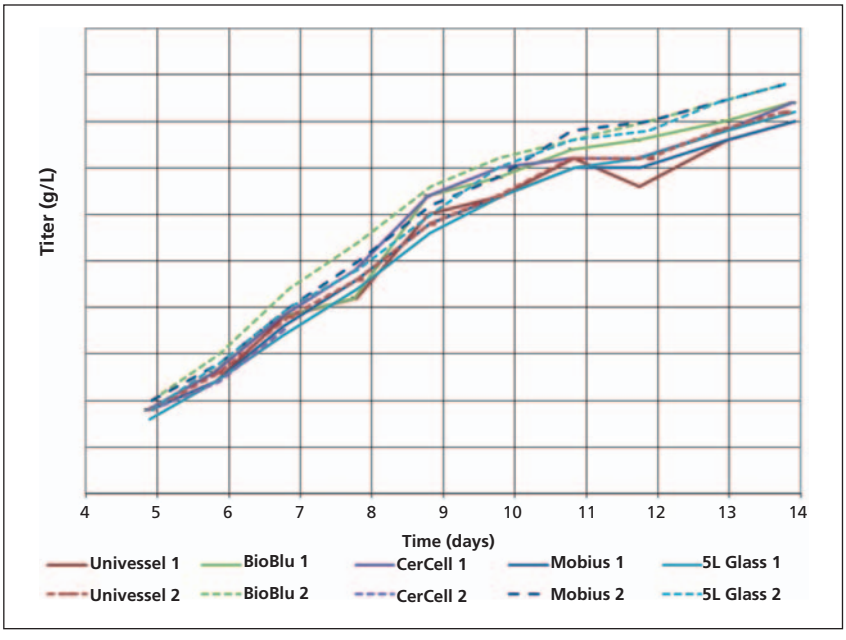
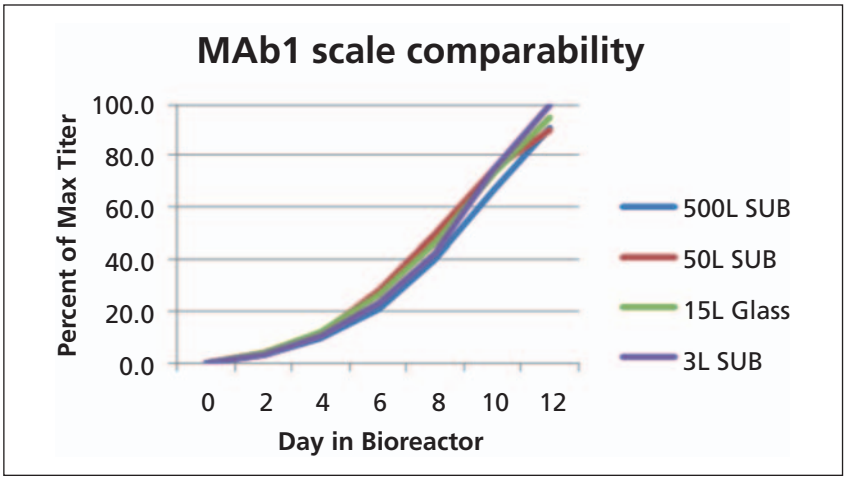


Figure 4. Product concentration vs. cultivation time for 3-L single-use bioreactor, 15-L glass vessels, 50-L, and 500-L single-use bioreactors for an antibody.



build-up in the bioreactor due do clogged air-filter was solved in different ways: UniVessel had two filters; BioBlu had a large off-gas filter (and possibility of Peltier cooling as an add-on); Mobius had a large-diameter tubing capable of removing condensate; and the CellVessel could be redesigned, if needed. All single-use bioreactor solutions for off-gas exhaust worked equally well in these experiments.

INTEGRATION BETWEEN MANUFACTURING SITES

Having standardized bioreactor solutions and designs is important both for small-scale (bench-top) and large-scale manufacturing applications when having multiple (global) manufacturing sites. Single-use bioreactors provide a standardized solution that can support a seamless transfer between sites when the same bioreactors are used at both ends. Additionally, eventual customization to bioreactor design can be used at all sites once produced at the supplier.

SCALE-UP AND CONCLUSION

An important consideration for implementing small-scale single-use bioreactors is scalability to large-scale bioreactors. This means the process can be developed and optimized prior to scale-up, and that troubleshooting or detailed process characterization campaigns, for example, can be carried out with a small-scale model that adequately represents the large-scale model.

Having standardized bioreactor solutions and designs is important both for small-scale and large-scale manufacturing applications.

The small-scale bioreactor process examined was scaled up to 2000-L scale, and it was found that with respect to cell-culture performance, metabolite profiles, product titer, and product quality, both the single-use bioreactors and the 5-L glass vessels provided good small-scale models of the large-scale model. The exercise of comparing small-scale single-use bioreactors to small-scale glass vessels and large-scale bioreactors has been done in several projects. Another example is shown in **Figure 4**, where product concentration for a 3-L single-use bioreactor, 15-L glass vessels, 50-L, and 500-L single-use bioreactors was compared for a project with an antibody again demonstrating good scalability between the small scales (single use and glass bioreactors) and production scale.

CMC Biologics' experience with this technology has been generated over recent years (1, 2). A novel approach with implementation of multiple 2000L bioreactors in either 3Pack or 6Pack configurations has been introduced (5). The Bioreactor 6Pack configuration (e.g., consists of six 2000L production bioreactors and a 2000L seed train) allows for flexible production with scales from 2000–12,000L in a single production suite. The bioreactors can be run in single unit operations or in groups, simultaneously, sequentially, or in staggered fashion to achieve desired production needs.

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Bridging the Gap Between Analytical and Process Protein A Chromatography

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EVENT OVERVIEW:

For decades, protein A has been synonymous with the process capture step in monoclonal antibody purification processes. An analytical protein A column is used for antibody titer determination of cell culture supernatants.

Due to the lack of a protein A analytical column with the same ligand as a process protein A resin, there is no direct link between upstream and downstream usage of protein A for the manufacturing of monoclonal antibodies.

With the availability from Tosoh Bioscience of a process protein A resin and an analytical protein A column with the same ligand, the gap between upstream titer determination and downstream purification of monoclonal antibody can finally be bridged.

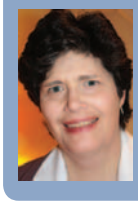
In this presentation a Tosoh Bioscience representative shows the process economic advantages of a new analytical protein A column with the same ligand as a process protein A resin. The seamless scalability reduces the amount of time for method development of monoclonal antibodies working their way through the product pipeline at both small and large biopharmaceutical companies.

Who Should Attend:

- QC HPLC analysts; Chromatographers; Method developers; Process engineers; Students

Presenter

J. Kevin O'Donnell, Ph.D.
Process Chromatography Support Manager
Tosoh Bioscience LLC



Moderator

Rita Peters
Editorial Director
BioPharm International

Key Learning Objectives:

- Learn how to evaluate a protein A ligand
- Understand how to link analytical and process scale protein A chromatography
- Discover the economic benefits of using the same ligand for both analytical and process scale protein A chromatography

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Perfusion in the 21st Century

Bruce Lehr and Delia Lyons

Quality, flexibility, and cost savings are driving use of perfusion technology in biosimilars manufacturing.

Reptile8488/E+/Getty Images

Cell-culture technology for biopharmaceutical manufacturing has steadily advanced over the past three decades. Batch processing, in which all raw materials are introduced into the bioreactor at the beginning of the cell-culture process, have largely been replaced with fed-batch systems, in which additional nutrients are added to maintain longer culture times. Now, improved continuous culture technologies such as perfusion are on the rise, as biosimilar manufacturers look for new ways to cut costs while increasing quality and productivity.

With perfusion technology, fresh medium is continuously introduced while spent medium, which generally contains the API and waste products, is removed. Cells are retained in the bioreactor by a device such as an alternating tangential flow (ATF) filter. If specific productivities are kept high, higher cell concentrations (generally, greater than 100 x 106vc/mL) can be attained resulting in greater volumetric productivity (more than 1.5 g/L day). Increased productivity can allow biosimilar manufacturers to achieve flexibility with a smaller facility footprint, and enable them to adapt operations for multiple products (1).

Perfusion was first introduced in the 1980s to enable the production of sensitive proteins that degraded when left in bioreactors under batch and fed-batch conditions. Around the same time, continuous processing was first recognized as a potential way to attain higher pro-

Bruce Lehr is director, and **Delia Lyons***, delia.lyons@sial.com, is senior scientist, both with MilliporeSigma's upstream R&D, cell culture.

*To whom all correspondence should be addressed.

ductivity than could be achieved via batch and fed-batch processes.

Equipment limitations, such as the lack of good aseptic connectors, marginal pump accuracy, and inadequate on-line monitoring, led to technical problems and regulatory uncertainties. There were questions about how to validate these processes, and how to define a batch that was produced under continuous operation (2).

Dramatic increases in the titers achievable with fed-batch culture, which reached levels as high as 4.7g/L (3), eventually eliminated the need to pursue continuous processing for increased productivity. By the early 2000s, perfusion was largely used to manufacture only products such as recombinant blood-clotting factors and enzyme treatments for rare metabolic storage disorders such as Fabry's disease, which are unstable in fed-batch systems. Perfusion is also used for biologics that are toxic to the producing cell line and cannot be produced under fed-batch conditions (4).

Within the past decade, however, changes in the biopharmaceutical industry and advances in perfusion technology have increased its rate of adoption. Many drugs have or will soon come off patent, increasing pressure on manufacturers to reduce costs. The biosimilars market is growing at a healthy rate (5), yet it is also extremely competitive.

Many manufacturers of branded biologics are shifting their focus from blockbusters to smaller-volume, targeted therapies such as orphan drugs. These drugs are often produced in smaller, more flexible facilities that are designed to manufacture multiple products, and often involve the use of disposable equipment. Biosimilars manufacturers are seeking technologies that can provide measurable competitive advantage.

CONTINUOUS BENEFITS

Continuous cell-culture processes (i.e., processes such as perfusion, that often operate continuously for 30–100 days) offer several advantages over more discrete traditional batch and fed-batch systems, which run for about 10–14 days. Constantly removing the product from the bioreactor reduces the retention time of the protein, a key improvement under production conditions that facilitate aggregation and degradation of the biologic active ingredient. In addition, the shorter retention times and more consistent conditions achieved at steady state provide more homogeneous product quality attributes (6).

Because of higher volumetric productivities, smaller bioreactors (i.e., 2000 L rather than 20,000 L) are more typically used for perfusion processes. The use of smaller bioreactors, even for commercial production, reduces the time and cost required for scale-up (7). Finally, perfusion can accommodate the shift toward multiproduct facilities, which often have different yearly production requirements (e.g., blockbuster versus orphan drug) while using the same size bioreactor, simply by running it for a longer or shorter culture time at steady state.

Similarly, changes in market demand can be met more rapidly, while use of disposable equipment reduces capital investment, cleaning and validation costs, as well as equipment footprint. Perfusion is also compatible with disposable technologies.

WHEN TO USE PERFUSION

Because fed-batch systems have been better known and are more familiar to date, many companies use them rather than perfusion systems. The exception has largely been production of sensitive proteins that degrade under fed-batch conditions. In some cases, perfusion has also been used to meet product quality targets such as N-glycan or sialylation profiles.

Today, numerous branded biologic and biosimilar companies are exploring the use of perfusion technology, often in conjunction with disposable equipment, to save costs. Notably, much higher peak cell densities (higher than 100 million cells/mL) can be achieved in perfusion than through fed-batch systems, and these high cell densities can be maintained for extended periods (i.e., from 30 to more than 100 days), resulting in higher product yields.

Perfusion processes are also seen as better suited to meet the needs of flexible, often single-use, multiproduct manufacturing facilities. Such manufacturing sites generally have smaller reactors and are faced with the challenge of preventing cross-contamination. Regulatory agency interest in the greater process control and product consistency afforded by continuous manufacturing (8) is also generating more attention around perfusion technology.

As perfusion process performance has improved, it has become increasingly possible for biopharmaceutical manufacturers to realize efficiency improvements. Notably, because of the sophistication and efficacy of the controls used for all aspects of perfusion processes, such as pumps with feedback control or advanced in-line and on-line analytical tools, it is now possible to monitor many of the key parameters that influence cell culture performance. Contamination issues have also been significantly reduced with the development of aseptic connections.

Cell separation devices have also become more efficient, allowing for very high cell densities to be achieved. It should be noted, however, that further developments in this area are needed, because it can

be technically challenging to work with the extremely high densities obtained in intensified perfusion.

Regulatory agency interest in the greater process control and product consistency afforded by continuous manufacturing is also generating more attention around perfusion technology.

Furthermore, protein retention has been reported when working at high densities with filtration systems. Manufacturers have access to more sophisticated media and more stable cell lines with new selection technologies that eliminate the need to use expensive selection agents that are required for selection agents such as the aminoglycoside, geneticin (G-418), 1-methionine sulfoximine (MSX), methotrexate (MTX), or puromicin. Once the selection pressure is removed, those cell lines tend to lose expression (9). The use of a glutamine synthetase (GS) system maintains the selection pressure having to add an agent.

IMPORTANCE OF MEDIA

While significant advances have been made in cell-culture media, there remains a need to optimize modified media for use in intensified perfusion processes. These media should be able to maintain the highest possible viabilities, cell densities, and specific productivities with the desired product quality at the lowest possible perfusion rates to minimize media consumption. Traditional media are not designed to overcome the buildup of toxins that can occur at these lower, cell-specific perfusion rates. Media designed for such perfusion processes must include not only a higher content of specific nutrients to maintain maximum performance, but also a modified balance of other nutrients to provide improved waste management.

Access to media designed specifically to address these needs is expected to have a significant impact on the adoption of perfusion in general.

The use of specialized media will lead to a measurable reduction in the cost of goods for drug production, with the added advantages inherent in perfusion of greater process consistency and improved product quality. As a result, the performance advantages of perfusion will be much greater than those of fed-batch. Once there is widespread recognition of these additional advantages of continuous cell culture, more branded biologic and biosimilar companies will likely select perfusion manufacturing for at least some of their processes.

PERFUSION SUPPORT

One of the challenges faced by all companies that are exploring use of perfusion processes is the limited ability to use high-throughput methods in perfusion mode. Currently, there is no perfusion device that can handle the working volumes typically used for high throughput techniques (1–200 mL). Industry efforts are underway to find alternative models that will extend the applicability of high-throughput methodology to perfusion to accelerate the media development workflow applied during custom projects (10,11).

While perfusion technology has been available for more than 30 years, it has been only in the past few years that branded biopharmaceutical and biosimilar manufacturers have expressed serious interest in its potential for biologic API production. Continuous cell culture offers many advantages, but in the end, for any given biotherapeutic, the choice between perfusion or fed-batch processing ultimately comes down to cost, if comparable product quality can be achieved using either technology. As a result, fed-batch is often preferred in existing plants with installed capacity, whereas in newer, multiproduct facilities, perfusion in single-use equipment provides favorable economics.

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Micro-Scale Bioreactors in Fed-batch Microbial Screening

Barney Zoro

The author outlines key functional attributes to assess a small-scale model.

Image courtesy of Sartorius

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Many bio/pharmaceutical companies develop genetically modified microorganisms to improve production of therapeutic proteins and industrial enzymes. Identifying the most productive and/or stable strains of these modified organisms prior to pilot-scale studies requires screening of large numbers in shake flasks and then further testing of the most promising strains in benchtop bioreactors. The need to perform time-consuming, resource-intensive experiments has resulted in the development of micro-scale bioreactor systems that offer a small-scale high throughput solution to accelerate microbial strain and fermentation development. This article describes the key functional attributes affecting performance of a micro-bioreactor mimic of benchtop bioreactors, enabling growth kinetics and protein expression to make the best early assessment of trends and lead strains.

In industrial fermentation, microbial cells such as *Escherichia coli* (*E. coli*) and *Pichia pastoris* can be genetically engineered to improve the production of therapeutic proteins and industrial enzymes. To further increase productivity and reduce costs, microbial screening studies are employed.

Microbial screening identifies stable strains that express high protein titers, as well as defines optimal media or culture conditions. This screening is typically performed in shake flasks or microplates, and a small number of the best performing microorganisms are then taken forward for evaluation in benchtop bio-

Figure 1: ambr 15 fermentation micro bioreactor for microbial screening.

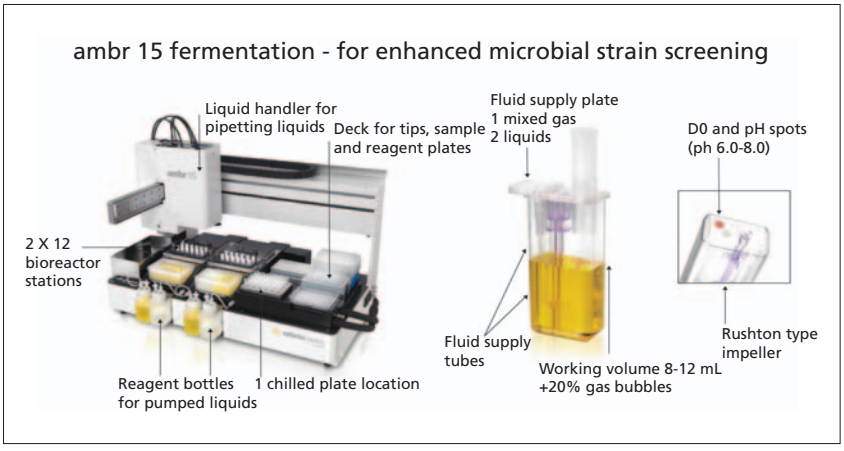


Figure 2: Oxygen transfer rates in a micro bioreactor.

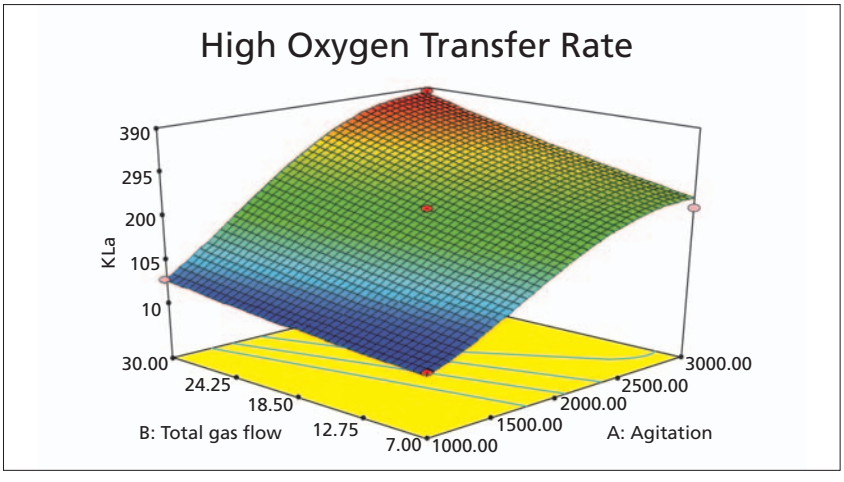
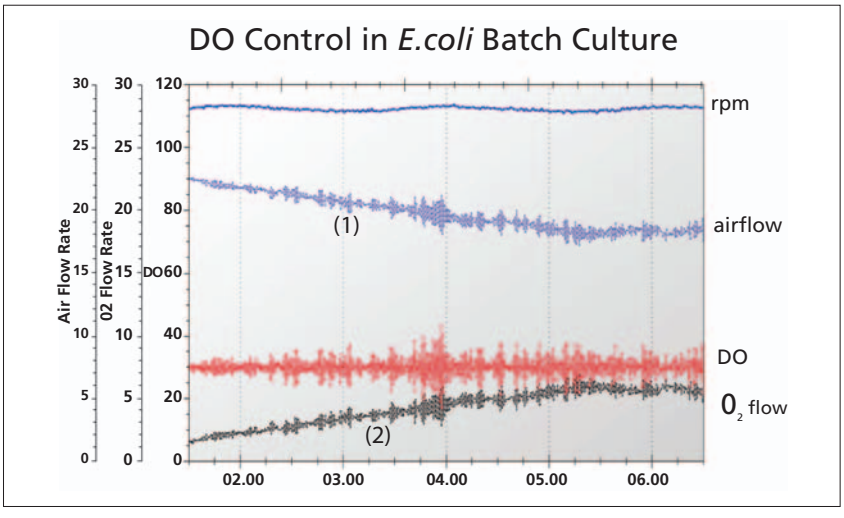


Figure 3: Dissolved oxygen (DO) control of *E. coli* batch cultured in a micro bioreactor.



reactors. The use of shake flasks and benchtop bioreactors is labor intensive and requires time-consuming pre- and post-process cleaning and sterilization for every experimental run. Because benchtop bioreactors often require a day to clean, sterilize, and set up, their screening use is limited to a small number of lead strains and can result in the final choice sometimes performing suboptimally upon scale-up. If a larger number of runs could be performed during microbial screening under conditions that are more representative of the pilot-scale environment, then it should be possible to isolate better performing strains for use in manufacturing, and potentially save thousands of dollars in cost of goods (COGs) while also reducing development time and costs.

DESIGNING A MICROBIAL MICRO BIOREACTOR

Microbial cells used for industrial protein production are generally robust and not susceptible to shear damage. This means that high efficiency radial impellers including the Rushton-type and relatively fast agitation rates can be used in manufacturing-scale microbial fermentation. The benefit of using these is that cell density and protein expression can reach its peak of productivity within 24 hours. The disadvantage is that oxygen and nutrients can be rapidly depleted by the fast growing cultures.

For microbial cultures, dissolved oxygen (DO) concentration is approximately 10–50% of air saturation for optimum growth. Therefore, sparging with air or pure oxygen and the addition of feeds are used in benchtop and pilot-scale bioreactors to maintain the cells in high-cell-density fed batch culture.

If a micro bioreactor is to accurately model a fed-batch culture in larger bioreactors, it has to include methods of stirring the culture, as well as supplying gases, acids, bases

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Figure 4: pH control of *E. coli* batch cultured in a micro bioreactor.

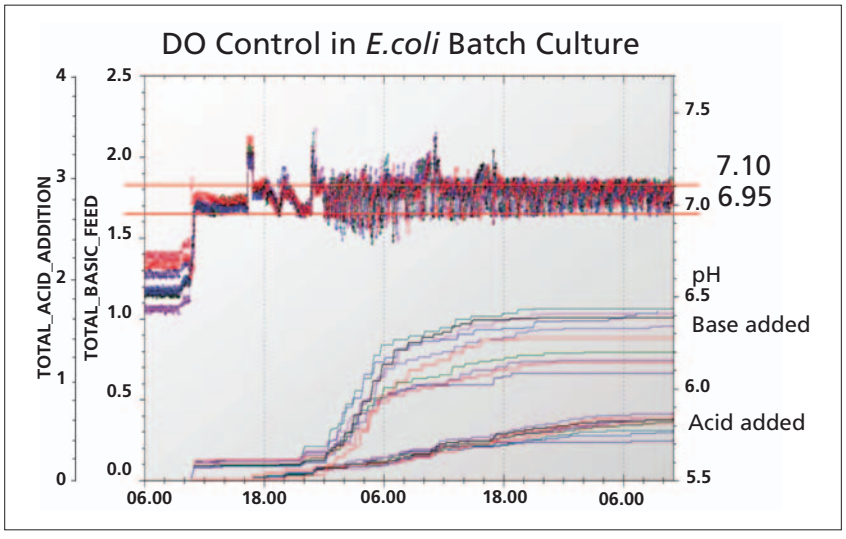


Figure 5: Cell density of *E. coli* cultured in a micro and a mini bioreactor.

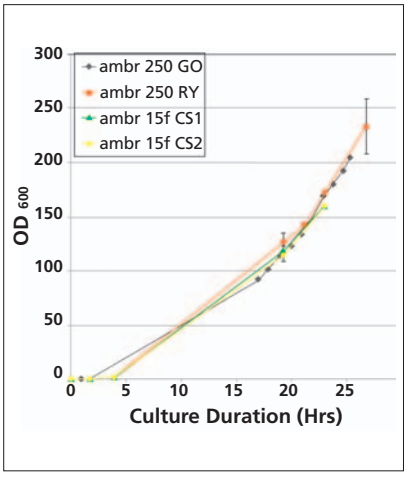
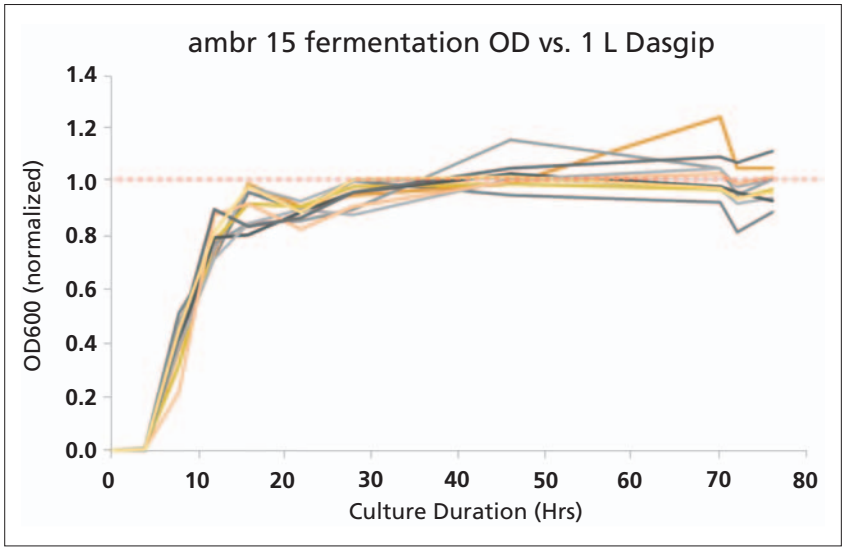


Figure 6: Cell density of *E. coli* cultured in a micro and a 1-L bioreactor.



and liquid feeds. These methods are required to maintain critical parameters such as oxygen supply and pH. The most important difference between batch screening development and production is the addition of feeds and maintenance of the culture as it reaches high cell densities during the fed-batch process. The author describes how these conditions were analyzed and how to determine the scalability of a micro bioreactor to benchtop bioreactors.

MATERIALS AND METHODS

A micro-scale bioreactor system (ambr 15 fermentation, Sartorius Stedim Biotech) was evaluated. The system combines 24, 8–12 mL working volume single-use bioreactors with an automated liquid handling workstation integrated to dedicated control and analysis software (**Figure 1**).

The micro bioreactor contains a Ruston-type impeller and has been designed with gas sparging capabilities to deliver high oxygen transfer rates. The workstation measures pH and DO every 12 seconds, and there are pumped liquid lines for base and feed additions in each micro bioreactor, enabling tight pH control and a semi-continuous feed supply.

EXPERIMENTAL APPROACH

Oxygen Transfer

The micro bioreactor's maximum oxygen transfer rate (the mass transfer rate of oxygen to the media) was determined using the sulfite oxidation method with water. A design of experiments (DoE) study was performed with a range of agitation speeds (1000–3000 RPM) and total gas flows in the range (7–30 volume per volume per minute [VVM]) to determine the maximum K_La.

DO and pH Control

An *E. coli* strain expressing a biopharmaceutical protein was cultured in proprietary media in 10 micro bioreactors to assess DO and pH control. A set point of DO 30%, with maximum agitation speed 3000 RPM and a maximum gas flow of 2 VVM were programed for each bioreactor via the automated workstation.

Contin. on page 32

Protein Impurities Pose Challenges

Cynthia A. Challener

Multiple methods are required for detecting and removing protein impurities.

Monty Rakusen/Getty Images

Residual undesired proteins—host-cell proteins (HCPs) and high-molecular-weight (HMW) and low-molecular-weight (LMW) species—have the potential to affect the safety and efficacy of biopharmaceuticals. As a result, the levels of these protein impurities in biologic drug substances and drug products must be controlled and are typically considered as critical quality attributes. The properties of the different contaminants vary significantly. In addition, the identification and quantification of some of these residual proteins, particularly in the presence of large concentrations of the product protein, can be challenging. Use of multiple orthogonal analytical methods and purification techniques is often required. Advances in mass spectrometry are, however, enabling more detailed analyses and thus improved monitoring of residual proteins during downstream processing.

The most common protein-based impurities that downstream processes are developed to control include high- and low-molecular-weight species, charge-variants, and host-cell proteins.

HMW species include dimers, trimers, tetramers, etc., formed of monomers that can be either covalently or non-covalently linked, according to John Moscariello, vice-president of process development for CMC Biologics. "Often, these large protein impurities consist of misfolded monomers in which surfaces of the monomer are exposed that typically would not be in the monomeric form. They are believed to primarily impact safety, but also can impact efficacy," he says. He does note, however, that there are data suggesting that some aggregated species are well tolerated. Even

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so, regulatory agencies expect biopharmaceutical manufacturers to control HMW species through specifications for the drug substance, drug product, and end-of-shelf life.

LMW species, which include clipped species and half molecules for compounds that are intended to be dimeric, such as monoclonal antibodies and bispecific antibodies, must also be controlled during downstream processing. These molecules can impact both safety due to the potential to cause immunogenicity responses and efficacy as the result of missing structural features, such as complementarity determining regions, which play a role in determining the antigen-binding specificities of antibodies and T-cell receptors, according to Moscariello.

HCPs are generated during the production of recombinant proteins. The host cells used for fermentation/cell culture produce HCPs necessary for the cells to function (growth, gene transcription, protein synthesis, etc.) and during cell death. They must also be controlled during downstream processing because they have the potential to elicit an immune response in patients due to the use of non-human cell lines (typically Chinese hamster ovary or CHO cells) for production. Some HCPs can also affect drug product stability. "Regulatory agencies expect robust control of these species through drug substance release testing," Moscariello observes.

MULTIPLE ANALYTICAL METHODS

The properties of the different types of residual protein contaminants in biologic drugs vary significantly, leading to the need to use different analytical methods to detect and monitor each. The presence of HMW species, for instance, is frequently determined using size-exclusion chromatography (SEC) coupled with high-pressure liquid chromatography (HPLC) or ultra-high performance liquid chromatography (UHPLC), while LMW species are detected using a capillary electrophoresis–sodium dodecyl sulfate (CE–SDS) (reduced and non-reduced) technique. HCPs are typically measured using enzyme-linked immunosorbent assays (ELISA).

"SEC–HPLC (or UHPLC) is a very robust assay and typically has very good resolution for HMW species, but it is often difficult to resolve LMW species," Moscariello says. As a result, CE–SDS is used for the detection of LMW protein impurities. The SDS denatures the protein, and if needed a reducing agent can be added to break any disulfide bonds. As in SDS–PAGE (polyacrylamide gel electrophoresis), the LMWs are separated based on size. For monoclonal antibodies, when running a reduced CE–SDS, typically highly resolved intact heavy and light chains, as well as any clipped species or non-reducible HMW species (i.e., covalently linked compounds) can be observed, according to Moscariello. To detect half-antibodies, however, non-reduced CE–SDS is frequently used because they are difficult to detect when using a reducing agent.

Although ELISA is the most common technique for monitoring HCPs, the selectivity of this method is dependent on the antibodies used in the ELISA kit, and consequently some HCPs can potentially be missed, according to Peter Levison, senior marketing director, downstream processing with Pall Life Sciences. HCP ELISAs employ a polyclonal mixture of anti-host cell proteins, but often do not provide complete coverage for all of the HCPs expressed during a specific process. This situation is particularly true for commercial kits. "While these kits allow for off-the-shelf detection of a wide range of HCPs for various cell lines, including CHO, they likely have significant gaps in HCPs for each specific process. As a result, ELISAs are known to show significant variability and it is difficult to get accurate values," Moscariello comments. On the other hand, he notes that the development of product-specific HCP assays with high coverage is costly and time consuming.

BETTER DETECTION

Most companies are exploring more advanced techniques for the detection of HCPs and other protein impurities that are designed to overcome the limitations of existing methodologies. In particular, Moscariello notes significant investment in process analytical technology (PAT) tools for online or at-line monitoring of HMWs and other contaminants. For instance, he points to the use by Amgen of multi-angle light scattering (MALS) to monitor protein aggregates at the effluent of a cation-exchange column.

Several advances in detection techniques for HCPs are also being made. Use of isotope tags for relative and absolute quantification (iTRAQ) HPLC technology can enable better detection of host-cell proteins, and when coupled with comprehensive genome sequences, can enable better identification of HCPs using established mass spectrometry (MS) databases, according to Levison. Data obtained from techniques such as surface-enhanced laser desorption/ionization-time of flight (SELDI–TOF) MS in combination with ELISA results are also allowing semi-quantification of total HCPs.

IMPROVED PURIFICATION

The first step in the purification of bioprocess fluids taken from bioreactors is clarification. In this step, the biologic-drug substance is separated from cell debris and turbidity is removed, leaving a clear harvested cell-culture fluid. This step is typically achieved via depth filtration or centrifugation and does also lead to the reduction of undesired protein impurities. Pall Life Sciences introduced the Cadence Acoustic Separator technology in April

2016—an alternative clarification method for cell-culture bioprocess fluids, including those from continuous perfusion processes—based on acoustic wave separation technology licensed in 2015 from FloDesign Sonics (FDS).

Following clarification, orthogonal chromatographic steps during protein purification are generally used to further reduce HCP, HMW, and other contaminants to acceptable levels, according to Levinson. "The most powerful step to remove HCPs is affinity chromatography," asserts Moscariello. There are issues with this method, however. While almost universally adopted for monoclonal antibodies, this method is often not applicable for non-fragment-crystallizable (Fc)-region-containing products. There are significant product-HCP interactions with antibodies. It is common to try to disrupt these interactions with harsh washes, particularly on affinity resins, which requires that they be salt-tolerant. Levison adds that the introduction of new mixed-mode modalities has provided additional selectivities that are providing improved HCP clearance.

There is also a lot of work being done with precipitating and flocculating agents, according to Moscariello. He notes that caprylic acid has been shown to significantly remove HCPs from product streams without significant loss of product.

FURTHER ADVANCES EXPECTED

Advances in detection technologies are having an impact on the capability of downstream processing to remove undesired protein impurities, according to Moscariello "The ability to identify specific HCPs using state-of-the-art MS techniques has allowed downstream development scientists to assess the charge, size, and hydrophobicity of these HCPs and exploit their differences from the product of interest and develop more robust processes," Moscariello adds.

"Improvements in analytics and genomic/proteomic analyses may well provide better awareness of the presence of contaminants and therefore facilitate their clearance," Levinson concludes. ◆

Bioreactors—*Contin. from page 29*

Growth Comparability

To determine if the micro bioreactor could generate comparable cell densities with a 250-mL and 1-L benchtop bioreactor, an *E. coli* strain expressing a biopharmaceutical protein was cultured in proprietary media in 10 micro bioreactors and a 1-L benchtop bioreactor (Dasgip). OD_{600} values for the micro-bioreactor cultures were normalized to the peak OD_{600} achieved in the 1-L bioreactor to enable publication of this industry data set. For comparison of growth between the micro bioreactor and mini bioreactors (250 mL, Sartorius Stedim Biotech), a non-expressing *E. coli* strain was cultured in proprietary media in fed-batch mode and the strain was cultured in the 250 mL mini bioreactor at separate laboratory sites at different times.

RESULTS AND DISCUSSION

The DO transfer data (**Figure 2**) shows that the micro bioreactor can achieve a maximum K_La of approximately 380 h-1, which is comparable to the oxygen transfer rate generated in 100 mL to 1L benchtop (1) and 14,000–20,000L pilot-scale bioreactors (2). The maximum K_La achieved indicates that the micro bioreactor can provide sufficient oxygen to equal or exceed the rate at which growing cells take up oxygen in larger scale bioreactors.

The DO control results (**Figure 3**) demonstrate that, in the micro bioreactors, the O2 flow rate increases to meet oxygen demand and the air flow rate decreases to maintain the 2 VVM maximum. The RPM and DO remained at their upper limits (**Figure 3**), and the DO is maintained at 30% during the batch phase of the culture.

The pH study (**Figure 4**) shows that the pH in the micro bioreactors is controlled to remain in the pH range of 6.95–7.1 throughout the 48 hours analyzed. A pH range of 6–8 is widely considered to be optimum for *E. coli* fermentation and means that the micro bioreactor can support cells in suitable pH conditions.

The scale-up data (**Figure 5 and 6**) demonstrate that the growth profiles from the micro bioreactors are consistent and all three bioreactor types support comparable cell densities. Additionally, high density cultures with an OD_{600} less than 150 can be achieved in the micro bioreactor, indicating that good predictions of large-scale, high density culture performance can be made.

CONCLUSION

A scale-down fermentation model needs to have a combination of high K_La (oxygen supply), tightly controlled pH, semi-continuous feed supply, and good mixing to provide consistent, reproducible, and predictive results. Data show that a micro bioreactor can achieve these requirements to effectively mimic a benchtop bioreactor in critical parameters such as DO transfer and pH control, as well as scalability of cell density from 10 mL to 1 L for fed-batch processes. Therefore, this type of bioreactor could be used in place of batch cultures such as shake flasks or microplates, significantly improving large-scale predictions of microbial screening studies. Better predictions of high cell density fed batch processes at the screening stage will enable the best strain choices, supporting rapid scale-up development of these high-density microbial cultures to produce therapeutic proteins and industrial enzymes in a shorter development time.

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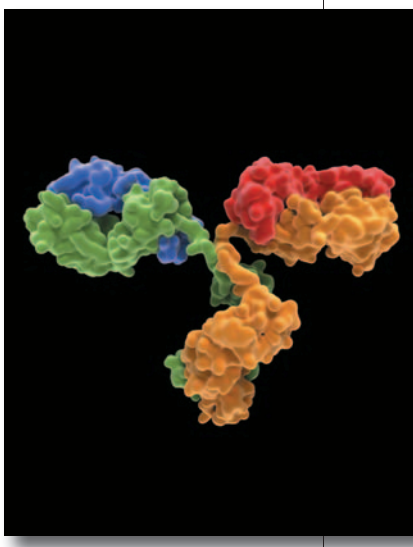
Enabling Freeze-Thaw Stability of PBS-Based Formulations of a Monoclonal Antibody

Part I: Freeze-Thaw Stress Testing



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Tatyana Mezhebovsky, Eric Routhier, Philip Sass, and Zahra Shahrokh

ABSTRACT

While phosphate-buffered saline (PBS) is commonly used in research and early development, significant freeze-thaw instability of PBS-formulated biotherapeutics has been established; crystallization of dibasic sodium phosphate is known as a major driver for degradation. The authors have identified an approach to stabilize PBS-based formulation for a monoclonal antibody (mAb). The effect of sodium chloride, nonionic surfactant, protein concentration, polyols, and freezing rates on freeze-thaw stability of a mAb was studied in 12 PBS-based formulations using six different freezing protocols. In formulations not containing polyols, aggregation, the primary degradation pathway, was observed for all freezing protocols, except freezing at -20 °C. Statistical analysis indicated high salt concentration as the most significant destabilizing factor followed by slow freezing at 1 °C/min, while higher protein concentration and polysorbate-80 had a stabilizing effect. Formulations containing polyols displayed no increase in aggregation for all freezing protocols. The antibody formulated in PBS containing polyols demonstrated stability on freeze-thaw stress at higher concentrations of both sodium chloride and protein. However, for polyol-containing formulations with low concentrations of both protein and salt, an increase in % polydispersity (due to submicron particle formation) was found by dynamic light scattering. The study demonstrates a systematic approach to stabilize PBS-formulated mAbs against freeze-thaw degradation.

Achieving biotherapeutic stability after freeze-thaw is an important deliverable of formulation development studies (1, 2). Routine practice in bioprocessing involves long-term storage of drug substance and of reference standard under frozen conditions to avoid chemical degradation (1, 3, 4). In addition, many protein reagents used in diagnostics and various assays in biotechnology are stored frozen and are often subjected to multiple freeze-thaw cycles during their lifetime (5).

A number of studies have been conducted to understand the parameters that affect freeze-thaw stability of protein solutions. Phosphate-buffered saline (PBS) is a common physiological buffer used in the discovery phase of formulating protein solutions in *in-vitro* and *in-vivo*

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PEER-REVIEWED

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Table I: Formulation compositions (A) and freezing protocols (B).
(A)

Formulation #	Composition			
	Protein concentration (mg/mL)	NaCl concentration (mM)	% Polysorbate-80	Polyol added
F1	5	150	0.01%	none
F2	5	150	0	none
F3	5	150	0.10%	none
F4	3	150	0.01%	none
F5	1	150	0.01%	none
F6	10	150	0.01%	none
F7	2.5	75	0.01%	none
F8	1	30	0.01%	none
F9	5	150	0.01%	5% sucrose
F10	5	150	0.01%	3% sorbitol
F11	1	30	0.01%	5% sucrose
F12	1	30	0.01%	3% sorbitol

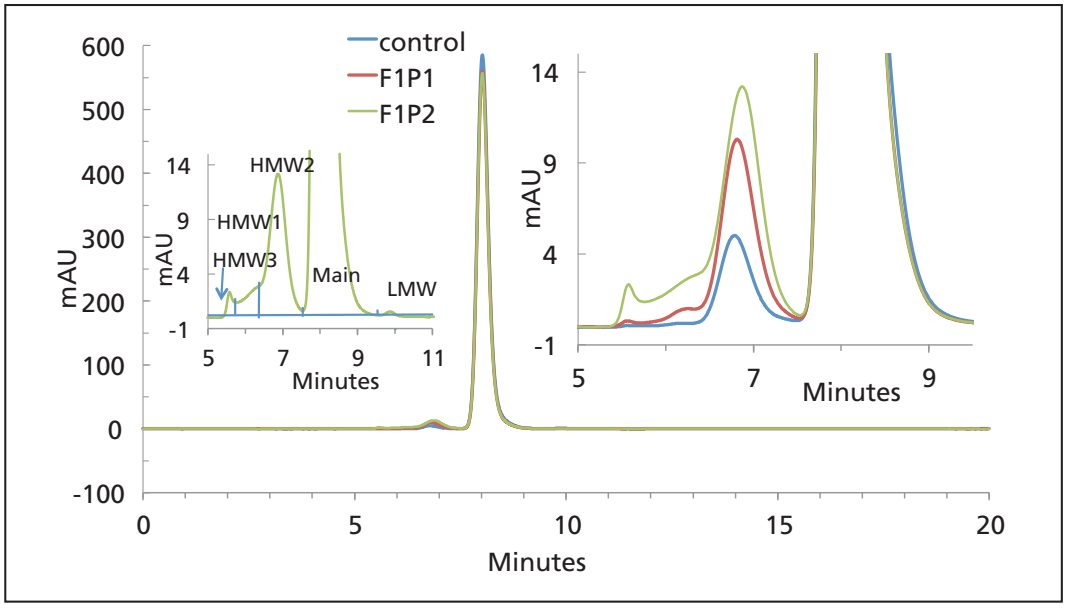
(B)

Freezing protocol	Sample volume	Freezing procedure	
		Condition	Temperature
P1	100 µL	Ambient	-80 ± 10 °C
P2	100 µL	CoolCell container*	-80 ± 10 °C
P3	100 µL	Liquid N_2 vapor	-80 ± 10 °C
P4	1 mL	Ambient	-80 ± 10 °C
P5	1 mL	Liquid N_2 vapor	-80 ± 10 °C
P6	100 µL	Ambient	-20 ± 5 °C
P7	100 µL	Ambient	2-8 °C
*Cools at 1 °C/min			

preclinical studies. The use of PBS, however, is avoided for biopharmaceuticals by formulation scientists because of protein freeze-thaw instability (6–8). The observed degradation of biotherapeutics after freezing and thawing in PBS is primarily attributed to a high propensity of sodium dibasic phosphate to crystallize, exacerbated by a pH drop of up to two pH units (8). Stabilization of proteins against aggregation after freezing and thawing by surfactant has been reported and believed to be because of the alleviation of interfacial protein denaturation at liquid-ice and liquid-air interfaces (9–11). While surfactants significantly mitigated aggregation on freeze thaw (9–11), a more complex effect, dependent on surfactant concentration, has also been observed (10). The effect of freezing and thawing rates on protein stability has been documented as well, where fast freezing/fast thawing has often resulted in minimal aggregation (6, 12). One study, however, observed a highly detrimental effect of flash freezing on the bioactivity of a model protein (13), while a recent study observed the most beneficial combination of fast freezing and slow thawing for preserving the activity of an enzyme at low concentrations (14). The effects of freezing and thawing rates are primarily attributed to the increase in solute concentration and the subsequent freeze concentrate effect on a protein's folded structure.

Given the common use of PBS in preclinical studies and the occasional selection of PBS in early clinical studies and most frozen

Figure 1: Representative size-exclusion high-performance liquid chromatography (SE–HPLC) profile of a monoclonal antibody (mAb) upon freeze-thaw stress.



diagnostic reagents, the authors conducted a systematic analysis of the freeze-thaw stability profile of a monoclonal antibody (mAb) formulated in PBS as a function of the vehicle composition and freezing protocol to identify potential stabilizing agents that would enable degradation-free frozen storage in PBS-based formulations. During the course of a three-part study, the authors assessed the stability profile of the mAb formulations after a single freeze-thaw stress (Part I), studied the long-term frozen storage stability of the formulation (Part II), and generated a mechanistic perspective of the observed effects using differential scanning calorimetry (Part III).

MATERIALS AND METHODS

The mAb used in the investigation is a recombinant mAb manufactured in Chinese hamster ovary (CHO) cells at Morphotek, Inc. and formulated in PBS (10 mM Na phosphate, 150 mM NaCl, pH 7.2). The formulation matrix and freezing protocols are listed in **Table IA** and **B**, respectively. Sample containers were 2.0-mL round bottom sterile cryogenic polypropylene vials. Samples for slow freezing and thawing protocols (P2 and P4) were placed in a CoolCell cell-freezing container (Biocision) for a highly reproducible (1 °C/min) cooling rate. Samples subjected to flash freezing (P3 and P5) were loaded into plastic cryogenic boxes and immersed into liquid nitrogen for 3–5 min prior to placement into a -80 °C freezer.

A single freeze-and-thaw process was conducted by freezing the samples overnight (12 h) and thawing for 8 h. All samples were thawed at 2–8 °C, except for samples that were flash frozen in liquid nitrogen vapor, which were thawed at ambient temperature (18–25 °C) to combine fast freezing with fast thawing. Product quality was analyzed by size-exclusion high-performance liquid chromatography (SE–HPLC) for soluble aggregates and dynamic light scattering (DLS) for submicron particulates. Duplicate samples were used for each protocol and each formulation. Analyses were initiated upon thawing and completed within two weeks, during which samples were stored at 2–8 °C.

The data were analyzed for significant effects using the screening model of the statistical software package JMP 10 (2012 SAS Institute) to identify the orthogonal factors that have a large statistically significant impact on the response (15). The level of aggregation was the response. The sum of three high molecular weight (HMW) species HMW1, HMW2, and HMW3 was used for the total soluble aggregate content. Relative aggregation, which was used as the response in the screening model, refers to the ratio of percentage aggregates post-freeze/thaw to percentage aggregates before freezing.

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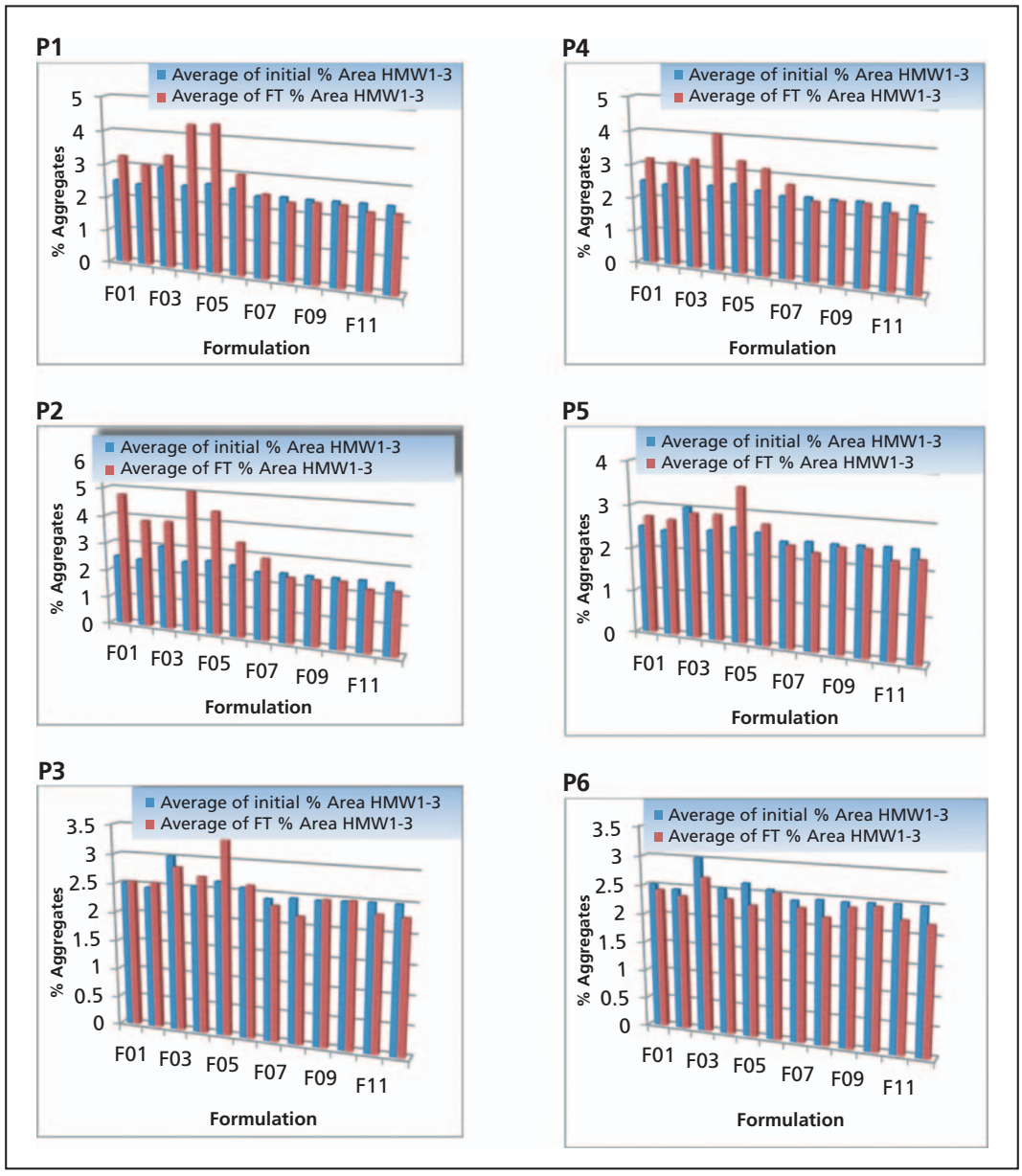
Figure 2: Effect of freeze-thaw on % aggregates in phosphate buffered saline (PBS)-based formulations of a monoclonal antibody. Aggregate level was measured by size-exclusion high-performance liquid chromatography. For each freezing protocol, the level of aggregates (Y-axis) before (left bar, blue) and after (right bar, red) freeze-thaw stress is plotted. HMW is high molecular weight.



Protein concentration, salt concentration, presence and type of polyols, freezing protocols, and presence of surfactant were the factors that were screened for effect on the response. Tonicifier content was denoted as 0, 1, and 2 for polyol-free, sucrose, and sorbitol in the formulations, respectively.

The screening results report "contrast values" (16), where the larger the contrast value of a parameter, the more that parameter affects aggregation. A bar chart plots the t-ratio, which is "contrast value" divided by pseudo-standard error. The larger the t-ratio, the greater the impact. Simultaneous p-value is analogous to the standard p-value for a linear model, but is adjusted to multiple comparisons (16). Significant parameter estimates were identified when p-value was <0.001.

Table II: Screening for major factors affecting stability of a mAb upon single freeze and thaw. The "effect size" is shown quantitatively by the "contrast" value in this JMP output and visually by the horizontal grey bars, which plot the t-ratio. The larger the effect size or contrast value of a parameter, the more that parameter affects aggregation. A positive effect size means the parameter promotes aggregation, whereas a negative effect size means the parameter stabilizes against aggregation. Data are sorted by the effect size. Significant factors are those with p value <0.05 and are marked with asterisk in the p-value column. Lenth's t-ratio is "contrast" divided by pseudo-standard error, which is an estimate of the residual standard error in the Lenth's method for identifying inactive (non-impacting) effects.

Term	Contrast	Plot of t-Ratio	Lenth t-Ratio	Simultaneous p-Value
Freezing Protocol	-11.1%		-10.0	<.0001*
tonicifier	-6.0%		-5.4	0.0003*
Freezing Protocol*[salt] mM	-5.9% *		-5.4	0.0003*
[protein] mg/mL	-4.7%		-4.3	0.0130*
% polysobrate	-4.3%		-3.9	0.0412*
[salt] mM*tonicifier	-4.1% *		-3.7	0.0648
Freezing Protocol*[protein] mg/mL*[protein] mg/mL*[protein] mg/mL	-2.0% *		-1.8	0.9998
Freezing Protocol*tonicifier*tonicifier	-1.8% *		-1.6	1.0000
% polysobrate*% polysobrate	-1.8% *		-1.6	1.0000
Freezing Protocol*[salt] mM*tonicifier*tonicifier	-1.7% *		-1.5	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol*Freezing Protocol	-1.7% *		-1.5	1.0000
Freezing Protocol*[salt] mM*[salt] mM	-1.6% *		-1.5	1.0000
Freezing Protocol*[protein] mg/mL*[protein] mg/mL	-0.9% *		-0.8	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol*tonicifier	-0.8% *		-0.8	1.0000
Freezing Protocol*Freezing Protocol	-0.7% *		-0.6	1.0000
Freezing Protocol*Freezing Protocol*[salt] mM	-0.6% *		-0.6	1.0000
Freezing Protocol*Freezing Protocol*[protein] mg/mL	-0.6% *		-0.5	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol*% polysobrate	-0.3% *		-0.3	1.0000
Freezing Protocol*Freezing Protocol*% polysobrate*% polysobrate	-0.2% *		-0.2	1.0000
Freezing Protocol*Freezing Protocol*tonicifier*tonicifier	-0.2% *		-0.1	1.0000
Freezing Protocol*Freezing Protocol*[salt] mM*[salt] mM	0.0% *		0.0	1.0000
Freezing Protocol*Freezing Protocol*[protein] mg/mL*[protein] mg/mL	0.0% *		0.0	1.0000
Freezing Protocol*Freezing Protocol*[salt] mM*tonicifier	0.2% *		0.2	1.0000
Freezing Protocol*Freezing Protocol*tonicifier	0.3% *		0.3	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol*[protein] mg/mL	0.4% *		0.3	1.0000
Freezing Protocol*Freezing Protocol*% polysobrate	0.4% *		0.3	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol*[salt] mM	0.9% *		0.8	1.0000
[protein] mg/mL*[protein] mg/mL	1.0% *		0.9	1.0000
[salt] mM*[salt] mM	1.3% *		1.1	1.0000
Freezing Protocol*% polysobrate*% polysobrate	1.3% *		1.2	1.0000
Freezing Protocol*% polysobrate	1.4% *		1.2	1.0000
Freezing Protocol*Freezing Protocol*Freezing Protocol	2.0% *		1.9	0.9997
[salt] mM*tonicifier*tonicifier	2.1% *		1.9	0.9986
tonicifier*tonicifier	2.2% *		2.0	0.9978
[protein] mg/mL*[protein] mg/mL*[protein] mg/mL	2.4% *		2.2	0.9823
Freezing Protocol*[salt] mM*tonicifier	3.4% *		3.1	0.3178
Freezing Protocol*[protein] mg/mL	4.9% *		4.4	0.0078*
Freezing Protocol*tonicifier	5.0% *		4.6	0.0048*
[salt] mM	9.9%		9.0	<.0001*

RESULTS AND DISCUSSION

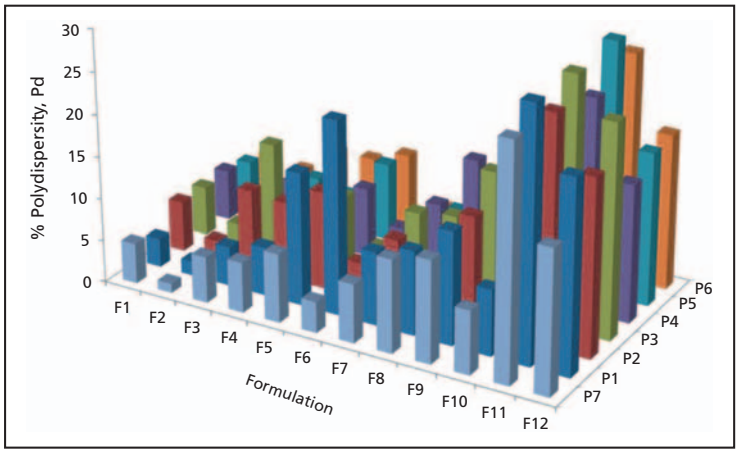
Aggregation profile

The SE–HPLC chromatogram of the studied mAb formulated in PBS displays three HMW species (see **Figure 1**). For this study, data analyses were conducted on the sum of three HMW species (HMW1, HMW2, and HMW3). Molecular weights of different association states of the mAb were evaluated by multi-angle light scattering (MALS) detection, which showed dimers for HMW2, hexamers for HMW1, and higher-order aggregates (approximately 24–25 monomeric IgG1 units) for HMW3. The HMW3 contribution to the total HMW content was drastically lower than that of HMW2 (see **Figure 1**).

Freeze-thaw stress

Figure 2 shows the level of soluble aggregates assessed by SE–HPLC before and after freeze-thaw stress test. An increase in aggregates was observed after a single freeze-thaw in all formulations, except those containing polyols (formulations F9, F10, F11, and F12) and F8 with low salt and low protein content. The data demonstrate significantly higher aggregation with a slower freezing than on flash freezing. For comparative analysis, the ratio of the post- to pre-freeze-thaw aggregate levels was modeled in the JMP 10 software to identify factors that significantly affect stability. The results of screening analysis are given in **Table II**, showing the significant effects of freezing protocol, NaCl

Figure 3: Effect of freeze-thaw on submicron particulates in phosphate buffered saline-based formulations of the monoclonal antibody. % polydispersity is measured by dynamic light scattering, bars representing each formulation and freeze-thaw protocol combination.



concentration, protein concentration, and P-80 concentration, with the highest contrast values, established as orthogonal factors and p-values at or below 0.01. Interactions between freezing protocol and NaCl concentration, freezing protocol and tonicifier, as well as freezing protocol and protein concentration were also observed. The screening results were consistent with the observed experimental trends, where the most detrimental factor was the slow freezing protocol (P2) and the highest level of NaCl, while polyols eliminated aggregation on freeze thaw. The screening modeling shows statistical significance of the experimentally observed effects.

An explanation for the detrimental effect of P2 (slow freezing and thawing rate 1 °C/min) might be a longer residence time at higher protein and excipient concentrations in the freeze-concentrate than that which might occur by more rapid freezing at -80 °C in P1 or flash freezing in P3. The beneficial effect of a lower NaCl concentration could be related to a lower salt concentration in the freeze-concentrate, while the beneficial effect of the higher protein concentration may be attributed to the smaller percentage of denaturation/degradation at the boundaries of the freeze-concentrate and other potential complex multiphase interfaces induced by freezing. A lesser protective effect of polysorbate-80 could be indicative of physical degradation occurring predominantly in the freeze-concentrate rather than at the ice-liquid and/or ice-air interfaces. The protective effect of polyols is in line with preferential exclusion of the polyols from the protein surface and enhanced hydration, as described by Arakawa and Timasheff (17). Select studies showed that sucrose in the range of 1%–5% and sorbitol in the range of 1%–3% prevented the aggregation of mAb after a single freeze thaw, so fine-tuning of polyol concentration can be easily incorporated into development of PBS-based formulations.

Surprisingly, no aggregation was observed on freezing at -20 °C (P6), while long-term storage at -20 °C was the most detrimental frozen storage condition (18). Since the aggregates of this antibody are not reversible at 2–8 °C, the two-week window during which analyses were conducted cannot be the reason for not seeing aggregation of the single -20 °C freezing/thawing process. Additionally, the SEC analysis was conducted consistently within the first few days after thawing, hence the erratic aggregation observed only over long-term storage of the antibody at -20 °C cannot be explained by the analysis window. It is possible that the solution super-cooled at -20 °C and did not freeze at baseline, hence generating similar results to P7 (liquid storage at 2–8 °C). It is more likely that super-cooling was accompanied by slow crystallization of NaCl, thus preventing aggregation on freeze-thaw stress by a kinetic effect. This hypothesis is in line with the long-term storage at -20 °C, where erratic aggregation was observed (18). Storage at -20 °C is near NaCl crystallization temperature as measured by DSC (19), thus protein aggregation is likely due to denaturation at the crystallized salt interface. The results for protocol P6 demonstrate limitations of freeze-thaw stress testing alone as a predictor of protein stability during long-term frozen storage at temperatures close to that of excipient crystallization or glass transition.

Submicron particles in the tested formulations were estimated by DLS. The DLS parameters calculated in secular mode with data fitted to a single mode distribution (monomer) generated % polydispersity (% Pd), a measure of the heterogeneity of the scattering particulates. A higher % Pd corresponds to a higher heterogeneity of the particle pool and hence a higher percentage of larger particles. The % Pd values for the freeze-thaw stressed samples are summarized in **Figure 3**. There was no effect of freezing on % Pd for any formulation or freezing protocol. Interestingly, formulations 11 and 12 containing low protein and NaCl concentrations in

the presence of polyols displayed the largest % Pd even prior to freezing, whereas formulations F9 and F10 containing a polyol in the presence of high protein and high NaCl concentrations had comparable % Pd as other formulations. The reason for such difference is not known; formulation F6 under protocol P2 also showed as high % Pd as F12, which was thought to be due to the presence of foreign submicron particles in that sample preparation.

The data suggest that addition of polyols to low protein and low NaCl containing formulations favors submicron particle formation, even when aggregation as detected by SE–HPLC is completely blocked. The results of the regularization fit (where the DLS data are fit to multiple pools of different types of particles) show that this higher heterogeneity in the low salt and low protein polyol-containing F11 and F12 samples is likely associated with a small number of large particles since most of the DLS signal intensity is correlated with monomeric antibody as reflected by negligibly low % intensity and % mass of the larger particles. As expected, there was no change in protein concentration of these formulations by absorbance at 280 nm. These results point to the necessity to complement analysis of aggregation with evaluation of submicron and subvisible particles using orthogonal methods such as DLS, microscopic flow imaging, and light obscuration (20). The mAb used in this study exhibited very low levels of subvisible particles throughout formulation development, hence analysis by methods such as microscopic flow imaging was not conducted because of material limitations. The data presented in the current study are consistent with the results published earlier on the specifics of freeze-thaw aggregation and particulate formation in phosphate-buffered formulation (21).

Overall, this study presents an approach for systematic evaluation of the effect of vehicle composition and freezing protocol on the stability of antibodies upon freezing and thawing. A sequel study (Part II) describes long-term frozen storage of the same PBS-based formulations when frozen under the same freezing protocols as described in this study (18). Analysis of both data sets together with the glass transition temperatures of the tested formulations (19) provide an understanding of the kinetic and thermodynamic processes involved in protein degradation upon frozen storage and identification of conditions for optimal long-term frozen storage stability.

CONCLUSION

The use of PBS as a physiologically suitable protein formulation was enabled by addition of polyols to overcome aggregation upon a single freeze-thaw condition. The most detrimental conditions leading to the IgG instability after freeze thaw were high NaCl concentration and slow freezing and thawing rates. On the contrary, increasing the protein concentration as well as addition of polysorbate-80 tended to improve freeze-thaw stability of PBS-based formulations. This systematic approach to optimizing PBS-based formulations could have applications in a broad range of biotherapeutics and life-science reagents.

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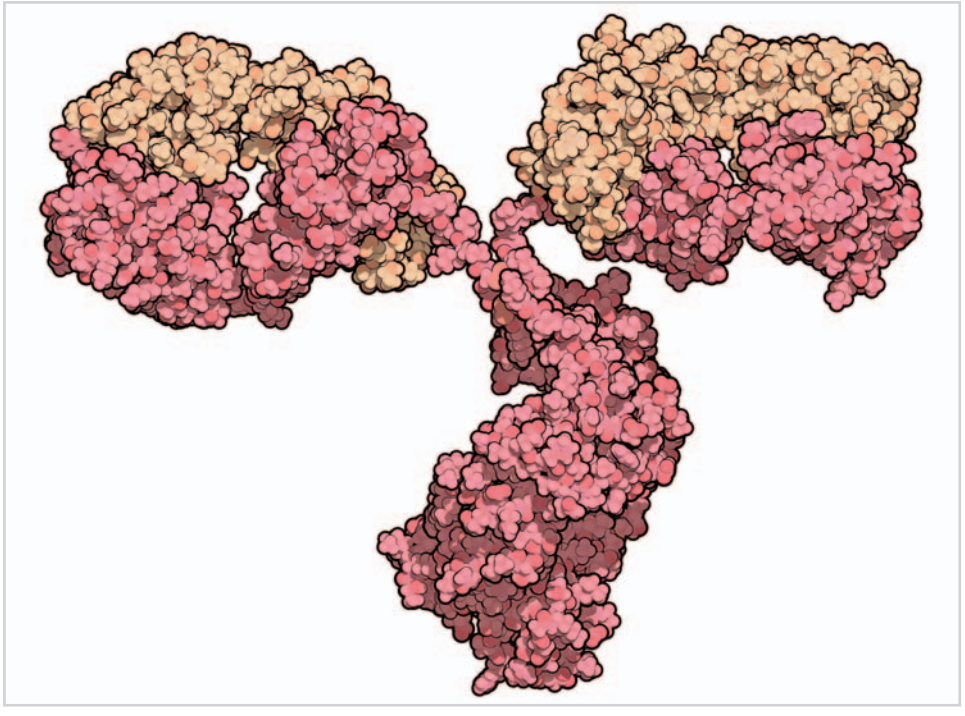
Generating a Fully Processed Antibody

Gregory Bleck, Dona York and David Rabuka

The authors outline cell-line development and process scale-up for an antibody program in which the antibody requires additional processing by a site-specific enzyme for correct functionality.



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The number of more complex biopharmaceuticals in development continues to increase. These include molecules such as virus-like particle (VLP) vaccines, proteins that require cleavage, or other additional post-translational modifications not typically performed by Chinese hamster ovary (CHO) cells, and more recently, numerous different multispecific antibody platforms. Each of these classes of molecules requires multiple proteins to be expressed at differing ratios to produce the correct biopharmaceutical. Many VLPs, for example, require two or more proteins to be produced in the right proportion to get the correct viral structure to mimic the virus itself.

In the case of a protein needing cleavage or another post-translational modification, the modifying protein needs to be produced at high enough levels to fully process all the biopharmaceutical at high titers without interfering with normal cell growth and the secretion of the molecule. Finally, some multispecific antibody platforms require three or more chains/subunits to be expressed at ratios that give the highest yield and the required structure for these molecules to be active.

Producing these multisubunit molecules or processing proteins in the correct proportions to obtain maximum titer and biopharmaceutical quality can be difficult using traditional cell-line development technologies. This is mainly due to problems getting different levels of protein expression for each of the multiple proteins needed for function and the ability of the system to

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obtain stable expression of each of those proteins over multiple generations in culture.

In this article, the authors outline cell-line development and process scale-up for an antibody program in which the antibody requires additional processing by a site-specific enzyme for correct functionality. Cell lines and processes yielding titers of 2.5–5.0 g/L of highly processed antibodies were generated, resulting in specific productivities of 40–75 picograms/cell/day.

CHARACTERISTICS OF CELL-LINE ENGINEERING TECHNOLOGIES THAT AID IN COMPLEX BIOPHARMACEUTICAL DEVELOPMENT PROGRAMS

Stability (genetic and expression)

Expression of all proteins required to produce the complex molecule needs to be stable through the cell-line development, subsequent scale-up, and eventually, large-scale manufacturing. Because balancing ratios of individual proteins is so crucial for these types of programs, any shift in amount of each pro-

Figure 1: Formylglycine-generating enzyme (FGE) protein recognition site and conversion process in the context of a recombinant protein of interest.

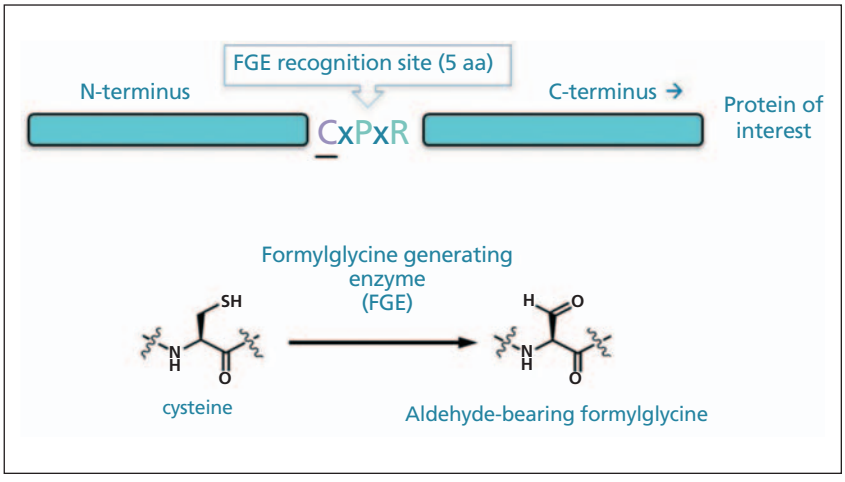

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Figure 2: Flow cytometry analysis of wild-type Chinese hamster ovary (CHO) cells and clones overexpressing formylglycine-generating enzyme (FGE). Mean fluoresce intensity (MFI) examined for each cell line.

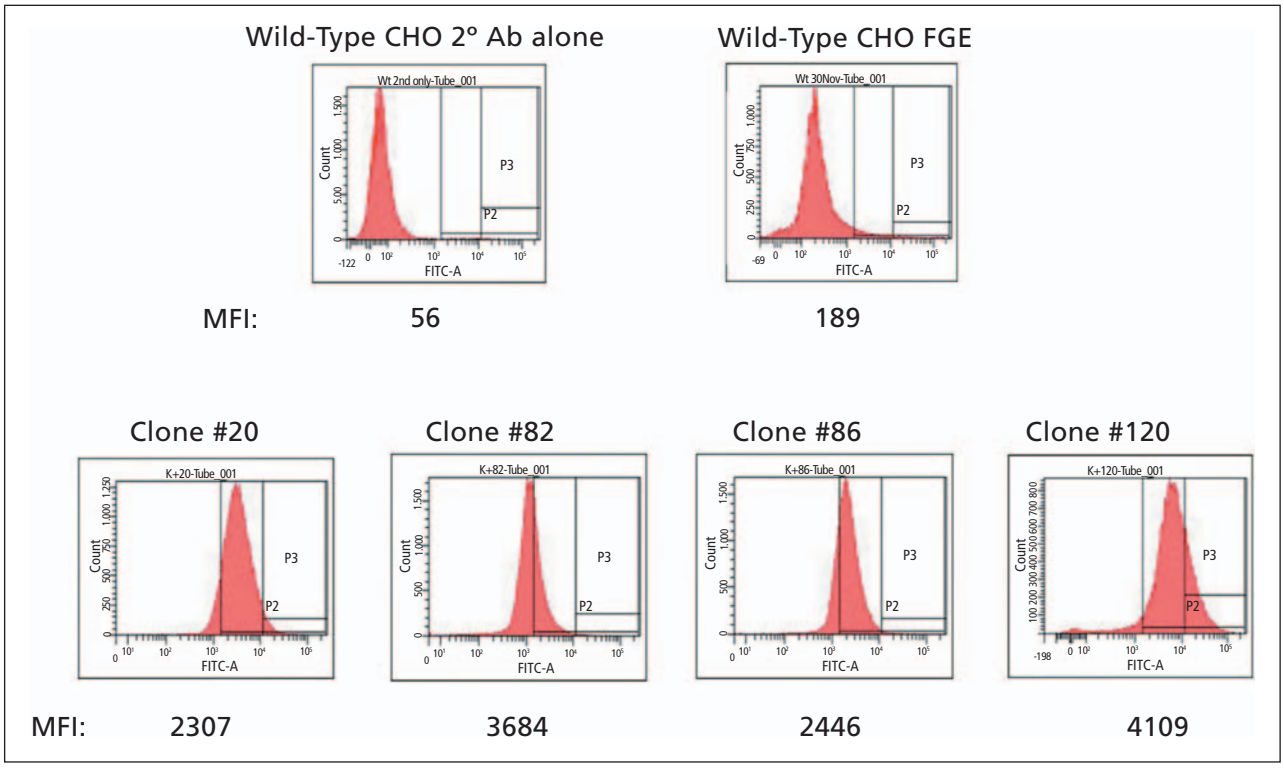


Table I: Formylglycine-generating enzyme (FGE) ELISA results from cell lysates for wild-type and FGE overexpressing cell lines.

Cell line	FGE (ug/mL of cell lysate)	FGE (pg/cell)
Wild-type CHO	0.18	1.75
Clone #20	17.01	170.17
Clone #82	29.41	294.23
Clone #86	35.62	356.34
Clone #120	156.68	1567.26

tein being produced during cell expansion and manufacturing can greatly impact the quality of the final product.

Ability to modulate ratios of protein expression

Different levels of protein expression are typically needed to maximize the production of a complex protein molecule. If a modifying enzyme is required in the process, a sufficient level of the enzyme is needed to fully modify the therapeutic protein; however, too much expression may affect cell growth or other cell behaviors. In the case of multispecific antibody platforms, certain unwanted chain pairings may occur more easily, prompting a desire to limit the production of that chain or subunit relative to the other ones being produced.

High gene insertion efficiency

The ability to efficiently insert genes into cells makes balancing ratios of protein expression a much easier process and shortens timelines. Complex protein molecules can require multiple cycles of gene insertion. Therefore, having a highly efficient gene insertion system resulting in a majority of cells containing the gene is important for both the protein product quality as well as shortening the project timeline.

Low-level use of selection markers

Many complex molecule programs rely on using numerous different gene constructs, each containing a different selectable marker. This use of multiple selectable markers in a cell line can complicate cell-culture processes, adding an increased burden on the cell-line machinery to produce multiple extra proteins. In addition, problems may arise during selection that affects the ability to identify clones that stably express the correct protein.

PROTEIN CONVERSION BY FGE

Formylglycine-generating enzyme (FGE) is an enzyme expressed in virtually all mammalian cells as well as most other eukaryotic and prokaryotic cell types (1). In the endoplasmic reticulum during protein synthesis, FGE performs the conversion of a cysteine (Cys) amino acid to a formylglycine

(fGly) amino acid. This conversion requires FGE expression in the presence of the consensus sequence, cysteine-X-proline-X-arginine, where X represents any amino acid other than proline. FGE catalyzes the oxidation of the Cys thiol to an fGly aldehyde. In normal cells, sulfatase enzymes are substrates for FGE and they require the consensus sequence along with the Cys to fGly conversion in their active site to be functional. Most any recombinantly expressed protein can be engineered to contain the FGE consensus sequence at single or multiple locations. Subsequent conversion of the Cys to the fGly results in a chemical handle (the aldehyde) that can be used for site-specific chemical conjugation (**Figure 1**) (2, 3, 4, 5). CHO cells produce endogenous active FGE; however, there is not enough FGE to perform full conversion of the Cys to fGly in proteins expressed with the consensus sequence, especially when the engineered proteins are produced at high titers. Also recently, the FGE enzyme was shown to require copper (II) for full activity (6, 7), so supplementation of copper (II) sulfate to the media optimizes amino acid conversion.

CELL-LINE ENGINEERING FOR HIGH-LEVEL PROTEIN EXPRESSION AND PROPER ENZYME CONVERSION

The goal of this case study was two-fold: to generate a stable CHO cell line expressing consistent high levels of FGE that could be used for a number of different programs, and to use the developed FGE cell line to produce cell lines and processes capable of expressing and converting high titers of tagged antibody. The FGE cell line needed to have all the normal phenotypic traits of the base CHO cells, but in addition produce enough FGE to allow full conversion of the tag at commercially viable titers and consistently do this over the num-

Figure 3: Non-optimized shake flask fed-batch production and conversion results of clonal cell lines producing the single tagged antibody. Tag site was located in the C-terminal end of the antibody heavy chain.

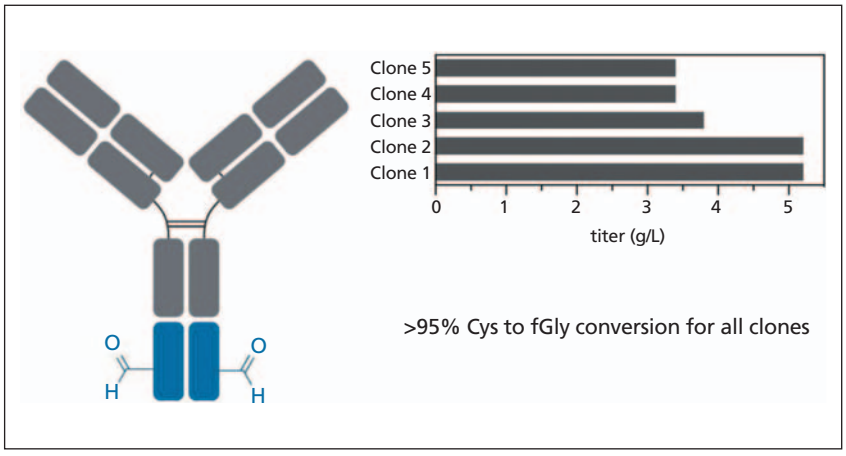


Figure 4: Non-optimized shake flask fed-batch production and conversion results of clonal cell lines producing the double-tagged antibody. Tag sites were located in CH1 domain of the heavy chain as well as at the C-terminus.

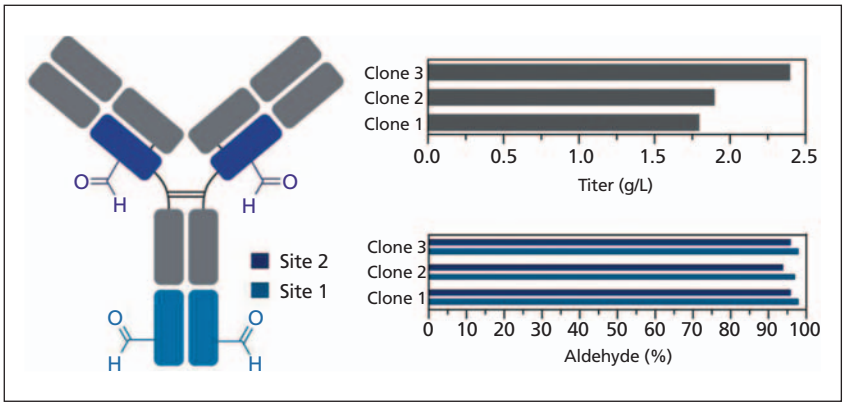


Figure 5: Duplicate 2-L bioreactor production runs under non-optimized conditions with a clonal cell producing the single tagged antibody. Specific productivities for both reactors were 34 picograms/cell/day.

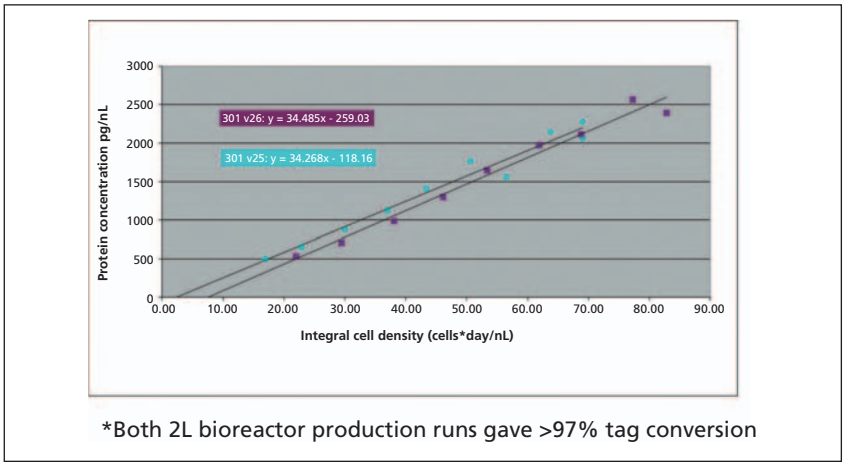
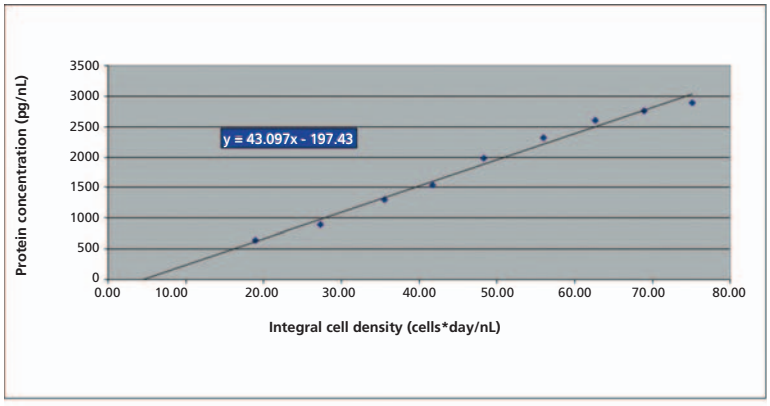


Figure 6: 100-L bioreactor production run under the non-optimized conditions with a clonal cell producing the single tagged antibody. Specific productivity for the bioreactor run was 43 picograms/cell/day.



*100L bioreactor production runs gave >97% tag conversion

ber of cell generations needed for a commercial process. The subsequently tagged antibody-producing cell lines were required to consistently produce fully converted antibody at titers greater than 1 gram/L in a traditional fed-batch manufacturing process.

The GPEx technology (Catalent) was used for all of the cell-line engineering. The GPEx method is based on the use of replication-defective retroviral vectors (retrovectors) to actively insert the desired genes into the genome of dividing cells. By controlling the number of retrovector particles accessing the cell, multiple gene insertion can be achieved without any of the traditional amplification steps (8). This was important for this study to obtain enough FGE production in the context of high antibody production.

DEVELOPMENT AND SELECTION OF HIGH-EXPRESSING FGE CLONAL CELL LINE

A master cell bank of our internal CHO cell line was the parent cell line for this work. The gene encoding human FGE was inserted into this line using the GPEx process. Two cycles of transduction were performed on the cells, and the resulting pool of cells was expanded for further development. Single-cell clones were isolated from the cell pool using limited dilution cloning. Approximately 120 clonal lines were initially screened. At the time, a high-throughput detection method for FGE expression was not available; therefore, clones were selected based on the number of copies of the FGE gene that were inserted into the genome of the cell line. No selectable markers are used as part of the process, so selection was based strictly on gene copy. Due to the possibility that overexpression of FGE could affect cell growth or protein function, clones were selected with a range of low to high gene copy numbers. Twenty-four clones were selected for further evaluation, eight with high FGE gene copy, eight with medium, and eight with low. The 24 clones were grown in culture for five weeks, and FGE level was analyzed using a flow cytometry-based assay at four different times throughout the culture. Based on FGE production and consistency of production over the extended culture, four clones were chosen for more detailed analysis (two high, one medium, and one low). These top four clones were again analyzed for FGE expression using the flow cytometry assay as well as a FGE ELISA method that was performed on cell lysates. The results indicated that one clone (#120) showed much higher expression in both assays compared with the other three clones (**Figure 2**, **Table I**). The high level of FGE expression did not significantly impact cell growth and doubling time for this clone, and it was selected as the lead candidate for future work.

EXPRESSION OF TAGGED ANTIBODIES IN THE FGE CLONAL CELL LINE

The selected FGE line was used as the base cell line to express two different antibodies. The two antibodies bind to different targets with one containing a single FGE consensus sequence and the second containing two sequences. The FGE line was engineered to produce the antibodies using the GPEx process. Limited dilution clonal selection was performed for each of the two programs and the top antibody-producing clones were selected for further analysis. These clones were evaluated in shake flasks under a nonoptimized fed-batch culture condition (containing copper [II] sulfate) measuring cell growth, antibody titer, and level of Cys to fGly conversion in the tagged sequences. Cultures were inoculated at approximately 300,000 cells/mL and harvested when cell viabilities reached approximately 50%. For the single tagged antibody, titers for the individual clonal cell lines ranged from 3–5 g/L, with specific cell productivities as high as 75 picograms/cell/day (**Figure 3**). Each of these clonal lines also showed greater than 95% Cys to fGly conversion. The clones producing the double tagged antibody gave titers ranging from 1.75–2.4 g/L, and both of the sites showed high conversion rates (**Figure 4**).

Contin. on page 50

Analysis of Glycosylation in Biosimilars

Richard Easton

A step-wise process is used to characterize glycans and understand the functioning of a molecule for biosimilar development.

Jeffrey Coolidge/Getty Images

The structures of protein drugs such as monoclonal antibodies are made more complex by post-translational modifications. The most notable of these is glycosylation, where carbohydrate residues are attached to the protein chain.

The variety of ways in which the monosaccharides in a glycan can be linked leads to a large diversity of structures that can be created from a limited number of building blocks. The pattern cannot be predicted from the genetic code, and the exact nature of the glycosylation depends on the cells and the conditions in which they are cultured. This adds an additional layer of complexity when proving biosimilarity as, even if the amino acid sequence is the same, the glycosylation pattern may be different, potentially altering the protein's biological properties. It is, therefore, important to use a cell line that produces an appropriate glycosylation profile or the same glycosylation profile as the reference material.

There are two main types of glycosylation commonly found on glycoproteins: N-glycosylation and O-glycosylation. To create N-glycosylation, the sugar residues are attached to the side chain of the amino acid asparagine, the initial step of the process being the transfer of a large, lipid-linked glycan donor to the protein. This immature glycan is composed of three glucose residues, nine mannoses, and two N-acetylglucosamine units. These residues are trimmed back to a core structure and rebuilt using other

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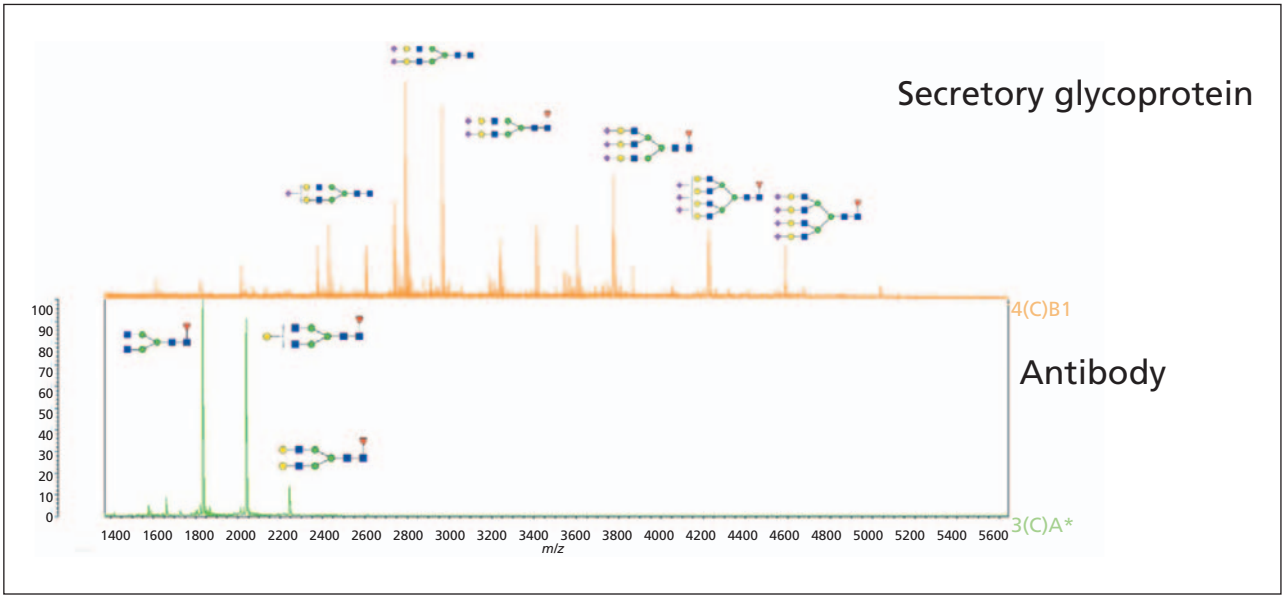
Figure 1. Matrix-assisted laser desorption ionization–mass spectrometry (MALDI–MS) analysis of released permethylated N-glycans.



monosaccharides that are attached via specific glycosyltransferases. This is where much of the variation arises: these transferases are a function of the genetic profile of the cell. Furthermore, the environment of the cell and the rate of protein synthesis will have an impact on the final glycosylation profile, also.

The process for the biosynthesis of O-glycosylation is different. Both serine and threonine residues can be glycosylated. First, a specific transferase attaches the monosaccharide N-acetylgalactosamine to the side chain of the amino acid. This is then extended into relatively short structures, typically only three or four monosaccharides long, using glycosyltransferases, some of which are identical to those involved in N-glycan biosynthesis.

The nature of the glycosylation process can result in significant heterogeneity in glycan structures. Nonetheless the glycans must be characterized in order to understand the functioning of the molecule, assess the outcome of the manufacturing process, and fulfill the requirements of the International Council for Harmonization's (ICH's) Q6B, *Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products* guidelines (1).

DETERMINING GLYCOSYLATION PATTERNS

Glycosylation pattern analysis is a step-wise process. As a simple first step, it may be enough to assess the type and amount of each monosaccharide composing the glycans. Typically, this involves breaking the sample down into its individual monosaccharides, which can be identified and quantified by the retention time and fragmentation pattern using gas chromatography–mass spectrometry (GC–MS).

If this information is combined with amino acid analysis to quantitate the amount of protein in the sample, the levels of different monosaccharides per unit protein can be determined. This is a simple test that gives a lot of information. For example, the presence of the monosaccharide N-acetylgalactosamine suggests the presence of O-glycosylation on the molecule. Fucose and galactose are both indicators of complex type glycosylation patterns. Monosaccharide analysis can also give a purity check: high levels of glucose might suggest the breakdown of Sephadex beads, for example, in the purification process, and high mannose levels may indicate yeast contamination.

With this information in hand, the next steps are to study the glycoprotein more closely to determine the structures of the glycans and where they are attached on the protein backbone. The intact glycoprotein must first be processed to make it more amenable to glycan release. To do this, first the disulfide bridges in the cysteine links are split to release free cysteine residues. These disulfide bonds are a key element in creating the 3D-structure of proteins and preventing them from unfolding; clearly, for further analysis, unfolding the protein from its tight globular conformation is advantageous. This is achieved via reduction and subsequent carboxymethylation to

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block the released thiol groups and prevent refolding.

The next stage is to use a protease to split the protein up into smaller peptide units, and this digestion is typically done using trypsin, but other enzymes can be used if trypsin is not appropriate. The peptides are then treated with the enzyme PNGaseF to cleave off the N-glycans from the molecule. On the rare occasion the protein has been generated in a plant or insect cell line, a different enzyme, PNGaseA, must be used instead. The released N-glycans are then purified. Any O-glycans are still present in the peptide fraction, and are then released via chemical reductive elimination and purified.

Finally, a permethylation procedure is performed to convert all of the free hydroxyl groups in the released carbohydrates into their methoxy derivatives. This leaves released, derivatized carbohydrates, which is advantageous in each of the three mass spectrometry-based analytical techniques used to determine the glycosylation pattern.

In matrix-assisted laser desorption ionization (MALDI)–MS, the use of permethylated glycans facilitates the clear identification of structures that would otherwise be very similar in mass and levels the playing field in terms of ionization, particularly for sialylated species.

Electrospray ionization–MS is used to look at the antennal structures of N-glycans. These are extensions of the core structures produced during biosynthesis. The fragmentation in this technique follows a handful of well-defined pathways, and the methyl groups greatly aid fragment identification. For example, the gal-alpha-gal epitope, which commonly causes immunogenic reactions, gives a signal at 668 Da.

The final analytical stage is GC–MS to identify how the monosaccharides are linked to one another.

Figure 2. Fragmentation in electrospray ionization showing formation of the A-type oxonium ion.

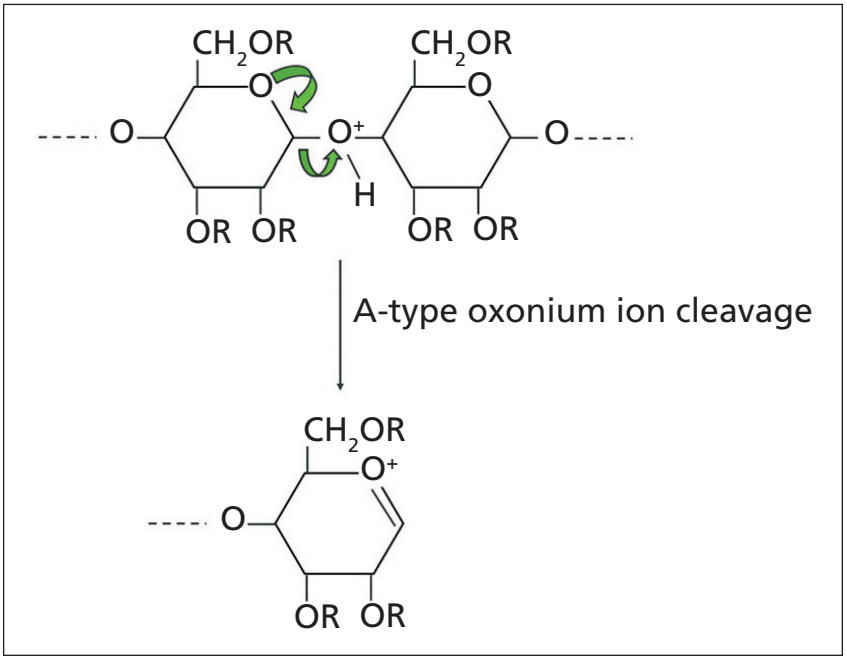
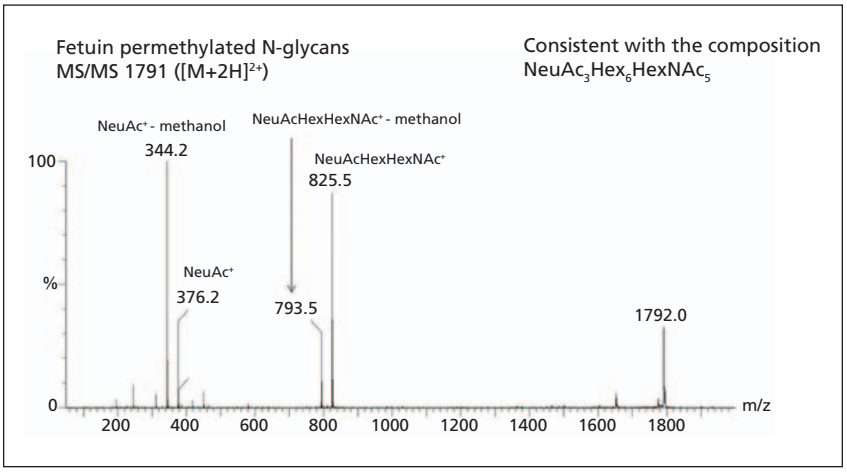


Figure 3. Nanospray mass spectrometry–mass spectrometry (MS–MS) analysis (performed on a quadrupole time-of-flight mass spectrometer) showing the fragment ions obtained from the selected mass. Both A-type fragment ions and subfragments generated by elimination of methanol from the A-type ions are observed.



For this analysis, the methylated glycans are hydrolyzed to their individual monosaccharides, and following several chemical steps, the newly released hydroxyl groups are acetylated. The GC–MS fragmentation patterns are definitive for identification of how the monosaccharide units are linked together.

ANALYTICAL EXAMPLES

An example of a MALDI–MS analysis of N-glycans is shown in **Figure 1**. This compares the MS traces of the N-glycans released from two different glycoproteins: a classic antibody and a secretory glycoprotein. Antibodies produce glycans that are relatively small

Figure 4. Linkage analysis of gas chromatography–mass spectrometry (GC–MS) spectra of 1,2-linked and 1,3,6-linked mannose as their partially methlyated alditol acetate derivatives. The fragment ion profiles generated are unique to different linkages of monosaccharide. GC/EI–MS is gas chromatography/electron impact ionization–mass spectrometry.

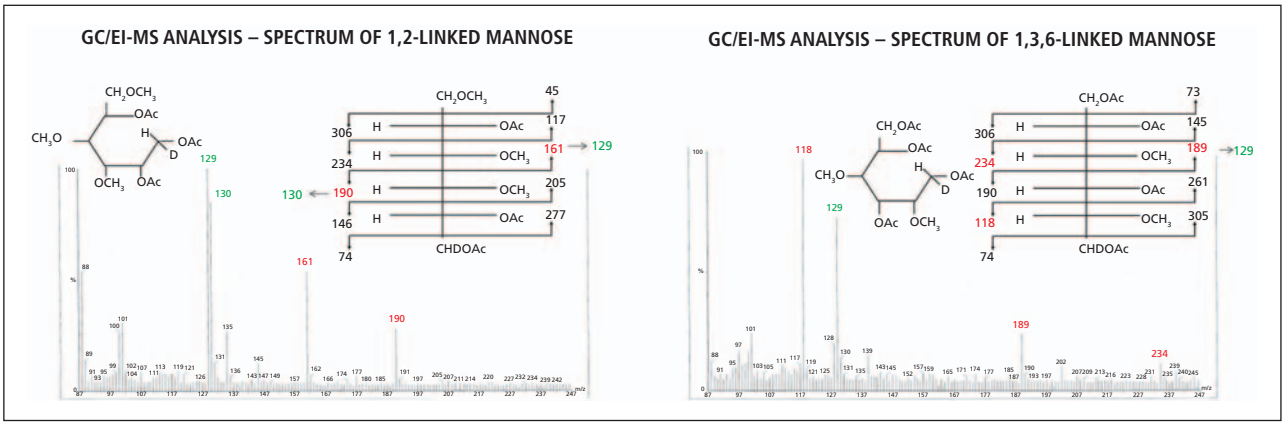
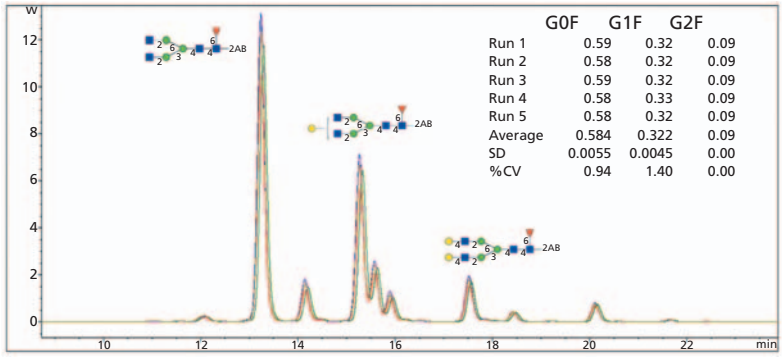


Figure 5. 2-AB stacked chromatograms of five separate N-glycan preparations of an antibody analyzed using hydrophilic interaction chromatography. The data demonstrate that reproducible glycan profiles are obtained, which can be used as comparators for other batches.



and simple compared to other glycoproteins because the two sites of glycosylation on the antibody's heavy chain are relatively inaccessible to the glycosyltransferases. Although the oligosaccharyltransferase will install the precursor, which is then trimmed back, it will only be rebuilt in a limited way. The result is two or three main structures, in this case the biantennary structures evident in the MS trace, with only relatively small masses.

In contrast, the trace for the secretory glycoprotein shows much larger glycans; this is typical of most non-antibody glycoproteins. The glycosylation sites are more exposed in these molecules, facilitating the ability of the glycosyltransferases to create larger and potentially more heterogenous structures, depending on the repertoire of transferases expressed in the cell. The mammalian cells that are typically used for the culture can produce up to four 'arms,', or tetra-antennary structures. These structures can be seen at the high mass (right) side of the upper mass spectrum in **Figure 1**. Structures with two (biantennary) or three (triantennary) arms can also be formed by the cell and these can be seen in the middle region of the upper mass spectrum in **Figure 1**.

While structures appear in the figure next to the peaks, it is unlikely that it would be possible to identify exactly what those peaks represent at this stage. While it should be clear that a particular mass represents, say, six hexose residues and five N-acetylhexosamines, their precise nature is not necessarily readily determined from the data. MALDI data gives compositional clues, allowing possibilities that are biosynthetically feasible to be drawn, but drawing definitive structures requires more data.

Figure 2 shows an example of a fragmentation pathway in electrospray ionization. As the carbohydrates have been permethylated, fragmentation is driven down a limited number of pathways, and one of the main pathways, the formation of the A-type oxonium ion, is depicted. This fragmentation mechanism produces a positive charge on the fragment ion, and this represents the non-reducing end of the molecule at which residues such as N-acetylglucosamine, galactose, fucose, or sialic acid are attached to form the anten-

nae. This fragmentation pathway occurs most readily on the reducing side of N-acetylhexosamine or sialic acid species; it does not readily occur on the reducing side of hexose residues.

By combining electrospray ionization with MS or MS/MS analysis in a quadrupole time-of-flight (Q-TOF) mass spectrometer, information can be gleaned about the different structural antennae that are present, allowing structures for the different combinations identified in the MALDI spectra to be pieced together.

An example MS/MS trace is shown in **Figure 3**. The instrument has been tuned specifically to one particular mass, which is fragmented, and the trace shows that fragmentation pattern. The use of selected energies in the source of the mass spectrometer allows for the formation of non-biased fragmentation of all the glycans that are present, and it allows compositions for antennal structures to be determined. It is still not definitive—whether a N-acetylhexosamine is N-acetylglucosamine or N-acetylgalactosamine is not clear, for example—but knowledge already built up about the biosynthetic pathway can be used to cut down the number of options.

Linkage analysis is used to determine how the monosaccharides in the glycans are attached to one another. **Figure 4** shows the difference between the GC–MS traces of 1,2-linked and 1,3,6-linked mannose. The starting point is those methylated structures, which are acid-cleaved to produce monosaccharides with free hydroxyl groups where the links were split. A reduction step converts the hexoses to linear carbohydrate chains to simplify the fragmentation pathway, and if a deuterated reductant is used, C1 in the chain will bear a characteristic deuterium atom in place of the hydrogen atom. This is a useful way of distinguishing the two ends of the chain, particularly if the structure is otherwise symmetrical. Finally, the molecule is acetylated, converting these newly produced hydroxyl groups to acetyl groups and resulting in the formation of so-called partially methylated alditol acetates.

These molecules will fragment down specific pathways in the GC–MS instrument. The methyl and acetyl groups are positioned differently depending on where the original linkages were, resulting in differences in the fragmentation pattern. These represent unique fingerprints for the structures, and the trace can be searched for the key fragmentation ions expected for the different linkages.

The final piece of the jigsaw is to take the MALDI, electrospray, and linkage data, and use them to construct biosynthetically possible glycan structures that meet all of the spectral requirements. It also highlights the potential presence of problematic immunogenic epitopes like gal-alpha-gal, which can be further confirmed by enzymatic digestion and subsequent MALDI–MS analysis of the products of digestion.

CONFIRMATION OF BIOSIMILARITY

Clearly, confirming biosimilarity runs deeper than simply characterizing structure, and chromatographic profiling of glycans provides a way to produce a pattern of the glycan population of the glycoprotein of interest. One such technique uses fluorescent tagging of released native glycans with 2-Aminobenzamide (2-AB) followed by hydrophilic interaction chromatography (HILIC). **Figure 5** shows 2-AB stacked chromatograms for five separate monoclonal antibody N-glycan preparations. The interaction of the glycans with the column matrix depends on the overall structure of the glycans, not just their mass.

The various antennary structures give different retention times, and thus the chromatogram provides a fingerprint profile. If the eluent is first passed through a fluorescence cell, and then into a mass spectrometer, information about the identity can be obtained, as well as the relative quantities of the different glycans in the sample. Chromatograms can then be assessed for similarity.

In summary, glycosylation is a post-translational modification present on many proteins of pharmaceutical significance, and as such must be characterized and monitored closely. MS analysis provides detailed structural information on the composition, antennal structure and linkage, while chromatography can be used to determine a unique glycan profile because of the interaction between different structures and the column matrix.

The use of specific proteolytic digestion procedures, in conjunction with chromatographic separation, allows the isolation of glycopeptides, which can then be analyzed using the techniques above to determine the structures of the glycans at individual sites within the molecule.

These are all important factors in proving biosimilarity. It is not enough simply to know what glycans are present on a protein: their positioning is equally important. The profile built up by careful analysis can be used as a reference standard against which batches can be tested to prove that they are, indeed, biosimilar.

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Syrris
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mAb Development—*Contin. from page 44*

PROCESS SCALE-UP TO BIOREACTORS

One of the single tagged cell lines was expanded for bioreactor production to confirm that the cell lines could be scaled with similar results. Duplicate 2-L bioreactors were seeded, and the culture conditions were identical to the shake flask study, except that the reactors were harvested at approximately 80% cell viability. The duplicate reactors gave similar results to each other with antibody titers of 2.5 g/L, specific productivities of 34 picograms/cell/day, and tag conversions greater than 97% (**Figure 5**). To confirm that the cell line and process could be run at a typical clinical production scale, the same cell line and process was repeated at the 100L scale. The cell line and process again showed consistent results with titers reaching 2.9 g/L, a specific productivity of 43 picograms/cell/day and greater than 97% tag conversion (**Figure 6**).

SUMMARY

Complex biopharmaceutical proteins in development are becoming more and more the norm with the advent of new antibody formats, unique VLP structures, and biopharmaceutical proteins that need additional processing. In the presented case study, a cell line needed to be developed that expressed three different genes (FGE, antibody heavy chain, and antibody light chain). Initially, a clonal cell line was selected that expressed FGE at high levels without impacting normal CHO cell characteristics. That base cell line was then used to produce two different tagged antibodies, both at commercially viable titers. FGE enzyme levels in those cell lines were capable of converting the antibody tag up to high specific productivities of 75 picograms/cell/day without impacting normal CHO cell behaviors in fed-batch culture conditions. Both the cell lines and culture processes were easily scalable to larger bioreactors while maintaining good antibody titers and the consistent tag conversion that is required for this unique site-specific conjugation technology, allowing for advancement into clinical development.

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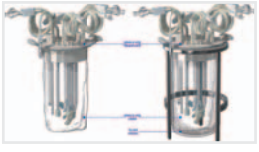
WuXi has started construction of a state-of-the-art, integrated R&D and manufacturing center at the company's headquarters (see press release). This 250,000 sq. ft. facility will be operational in 2017 and can accommodate 800 scientists. The center will provide comprehensive concept to clinic biologics discovery, development, and GMP manufacturing services on one consolidated campus. This facility utilizes WuXi's single-source technology platforms. **WuXi Biologics (A wholly owned subsidiary of WuXi AppTec),** info@wuxibiologics.com, www.wuxibiologics.com/news-events.



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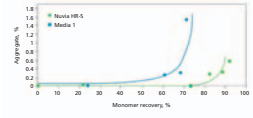

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Page header image: Arthur S. Aubry/Getty Images

IN THE PIPELINE

NIH-Backed Yellow Fever Vaccine Trial Announced

The National Institute of Allergy and Infectious Diseases (NIAID), part of the National Institutes of Health (NIH), announced it has begun a Phase I clinical trial of an investigational vaccine to protect against yellow fever virus. The experimental vaccine is being developed by the Danish biopharmaceutical company Bavarian Nordic. A live attenuated virus vaccine for yellow fever has actually been in existence since the 1930s, but the Centers for Disease Control and Prevention (CDC) reports that no vaccine efficacy studies have been performed with the yellow fever vaccine, also known as 17D.

Although 17D is manufactured worldwide at the Bio-Manguinhos Oswaldo Cruz Foundation in Rio de Janeiro, Brazil; the Institute of Poliomyelitis and Viral Encephalitides in Moscow, Russia; the Pasteur Institute of Dakar in Dakar, Senegal; Sanofi Pasteur in Lyon, France; and at Berna, in Berne, Switzerland (formerly produced at the Robert Koch Institute in Berlin, Germany), the only 17D yellow fever vaccine approved for use in the United States (known as YF-Vax) is manufactured by Sanofi Pasteur in Swiftwater, Pennsylvania, according to the CDC. Only four of the six vaccine manufacturers are prequalified by the World Health Organization (WHO) to distribute vaccine product internationally. At a meeting earlier this year, WHO called the recent reappearance of yellow fever a serious health concern.

Manufacturing and supply issues have limited the amount of 17D that is available on the market. Plus, because the current vaccine options are made from a live attenuated virus, the vaccine cannot be used to treat certain immunocompromised patients. Current options, such as Sanofi's Dengvaxia (CYD-TDV) and Imojev (ChimeriVax-JE) yellow fever vaccines, are made from live attenuated versions of the virus.

The NIH-backed vaccine candidate from Bavarian Nordic (MVA-BN-YF) will also be based on a weakened version of a virus, but will use a strain of the Modified Vaccinia Ankara (MVA) virus as a vaccine vector to carry yellow fever virus genes into the body. The MVA smallpox vaccine Imvamune is a non-replicating, highly attenuated live vaccinia virus. According to Bavarian Nordic, more than 7600 people, including 1000 individuals who are immunocompromised, have been safely vaccinated with vaccines using their MVA-BN-based platform. The company is currently in a partnership with Janssen to develop an Ebola vaccine using the same vaccine model.

Kite Collaborates with UCLA to Develop Off-The-Shelf Allogenic T-Cell Therapies

On July 25, 2016 Kite Pharma entered into an agreement with the University of California, Los Angeles, (UCLA) to advance development of off-the-shelf allogenic T-cell therapies from renewable pluripotent stem cells. The company entered into an exclusive license agreement with UCLA for an artificial thymic organoid (ATO) cell culture system. The ATO replicates the human thymic environment to support efficient *ex vivo* differentiation of T-cells from primary and reprogrammed pluripotent stem cells.

The technology is based on research led by Gay M. Crooks, MD, professor in the Department of Pathology and Laboratory Medicine and the David Geffen School of Medicine at UCLA. The ATO system has the potential to support scalable production of T-cells using pluripotent stem cell lines capable of indefinite self-renewal.

Under the terms of the agreement, Kite will receive exclusive rights to use the licensed technology to develop and commercialize T-cell products in oncology, the company noted. In connection with the license agreement, Kite has entered into an agreement with UCLA to support ongoing preclinical research in Crooks laboratory to optimize the ATO platform.

Bristol-Myers Squibb and AbbVie Team Up on Cancer Trials

Drugmakers AbbVie and Bristol-Myers Squibb (BMS) will be teaming up to evaluate the efficacy of AbbVie's investigational biomarker-specific antibody drug conjugate Rova-T (rovalpituuzumab tesirine) in combination with BMS's Opdivo (nivolumab) and Opdivo + Yervoy (ipilimumab) regimen as a treatment for relapsed extensive-stage small cell lung cancer (SCLC).

According to the companies, the Phase 1/2 clinical program will explore the potential of combining BMS' immuno-oncology agents, which are designed to alleviate immune suppression, in conjunction with AbbVie's investigational antibody drug conjugate, Rova-T, to drive improved and sustained efficacy and tolerability. Rova-T is a novel antibody drug conjugate that targets and eliminates tumor initiating cells and other bulk tumor cells. This collaboration will determine if the targeted cell killing and antigen release caused by Rova-T may further enhance the effect of immunotherapy. Opdivo is a PD-1 immune checkpoint inhibitor, and Yervoy is a CTLA-4 immune checkpoint inhibitor for patients with unresectable or metastatic melanoma.

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post-approval manufacturing changes that can be documented in an annual report that was issued in March 2014 (2). This new revision reiterates the concept of like-for-like replacement while expanding some of the facility changes that can be made as an annual report. For instance, the new guidance states, "For equipment used in aseptic manufacturing processes (e.g., new filling line, new lyophilizer), replacement of equipment with that of the same design and operating principle, when there is no change in the approved process methodology or in-process control limits" ... and ... "In the manufacturing of sterile products, the addition of barriers within a conventional fill area to prevent routine in-process human intervention in an existing filling or compounding area that is qualified and validated by established procedures" are acceptable changes to include in an annual report. Many pharmaceutical professionals would have considered these changes as needing prior approval before being implemented. Be cautious, though; just because these improvements can be managed through the annual report process, you still need to do all the necessary work to ensure the change is appropriate and does not affect product quality.

COMPARABILITY PROTOCOLS

There may still be some clients wary of the change even when you notify them of the change and the guidelines that support making the change without going through the PAS process. This is where the third guidance comes into play, and it is the updated guidance on comparability protocols for human drugs and biologics (3). A comparability protocol has the potential to decrease the filing category. For instance, items that would normally be handled as a PAS have the potential to be considered as a changes-being-effected-in-30-days supplement. By employing the use of a comparability protocol you are, in essence, making sure your client understands the change you will be implementing and the data you will be collecting and reviewing to assess that the upgrade was successful and did not affect product quality. The comparability protocol is a nice compromise when you have clients insisting on a PAS and others who are comfortable with the annual reportable strategy. It allows you to file the protocol as a PAS and build the inventory needed in preparation for the anticipated shutdown period.

There are no right or wrong answers when recommending filing strategies to a client. Regardless of what the contract manufacturing organization says, the client can always chose an alternative. The best way to convince a client of a filing strategy is to make sure you have a robust quality agreement in place that gives you the responsibility for maintaining your facility. Become familiar with the regulations and use them to justify your recommendation. Finally, use a comparability protocol for proving like-to-like equivalency, and present the recommendation to the client in a documented manner. These steps should help you implement a facility upgrade in a timely manner while reducing your downtime to make the improvement.

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Ad Index

Ask the Expert

One Strategy for Updating an Aging Facility

Susan Schniepp, distinguished fellow at Regulatory Compliance Associates, discusses the regulatory requirements for improving manufacturing lines.

Susan Schniepp is distinguished fellow at Regulatory Compliance Associates.

Q: I work for a contract manufacturing organization (CMO) and my management has dedicated capital funds to update some of our manufacturing lines. I need to work with my clients to get approval to make the necessary changes. Many of my clients feel any changes to the manufacturing line would require a prior approval supplement (PAS), which would be a challenge for our company. Do you have any advice regarding a regulatory strategy that I could propose to my clients that would satisfy them and minimize downtime?

A: This is a great question and a complicated issue. The pharmaceutical industry has been trying to become more efficient from both manufacturing and regulatory perspectives. The challenge is to improve processes, quality systems, and manufacturing capabilities while operating efficiently and in a manner that ensures safe, effective, and cost-efficient medicines to patients. Many updates to processes and quality systems can be easily and readily implemented with little or no impact on regulatory filings. When a change impacts regulatory filings, it has the potential to disrupt the supply chain if it is not handled appropriately and as efficiently as possible. FDA seems to recognize these situations and has been working to help lessen the regulatory filing burden to companies while not affecting the quality of the products. In the past three years, FDA has issued three important guidelines to help facilitate the dialog between clients and CMOs (1) and expedite the regulatory post approval change (2, 3).

QUALITY AGREEMENTS

The first step in the process of upgrading your facility is to get the agreement or, at least, an acknowledgment from your clients that they are aware that you intend to upgrade the facility and that it may affect their regulatory filing. The first guideline that helps you facilitate the dialog with your client is the quality agreements guidance issued in May 2013 (1). This guideline states "... FDA recommends that owners and contracted facilities implement written quality agreements as a tool to define communications, delineate responsibilities, and assure the quality, safety, and effectiveness of drug products." If you have a quality agreement, then you need to check and see whether the contract organization or the client has the responsibility to maintain the facility. If you have relegated this responsibility to your clients and they are hesitant to implement the upgrade, remind them that the quality agreement guideline gives you the authority to maintain your facility (including necessary equipment upgrades) by stating "A quality agreement does not exempt contracted facilities from CGMP requirements related to the operations they perform, regardless of whether such CGMP requirements are specifically discussed in the quality agreement." Bottom line, both you and your client(s) may have responsibilities outlined in the quality agreement regarding maintaining and upgrading the facility but you, as the CMO, need to adhere to CGMPs and make sure your facility is maintained per 21 *Code of Federal Regulations (CFR)* 211.58 requirements that "any building used in the manufacture, processing, packaging, or holding of a drug product shall be maintained in a good state of repair."

CMC POST-APPROVAL CHANGES

The second guideline that helps in updating your facility in a timely manner is the chemistry, manufacturing, and controls (CMC)

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