GEN Genetic Engineering & Biotechnology News eBook

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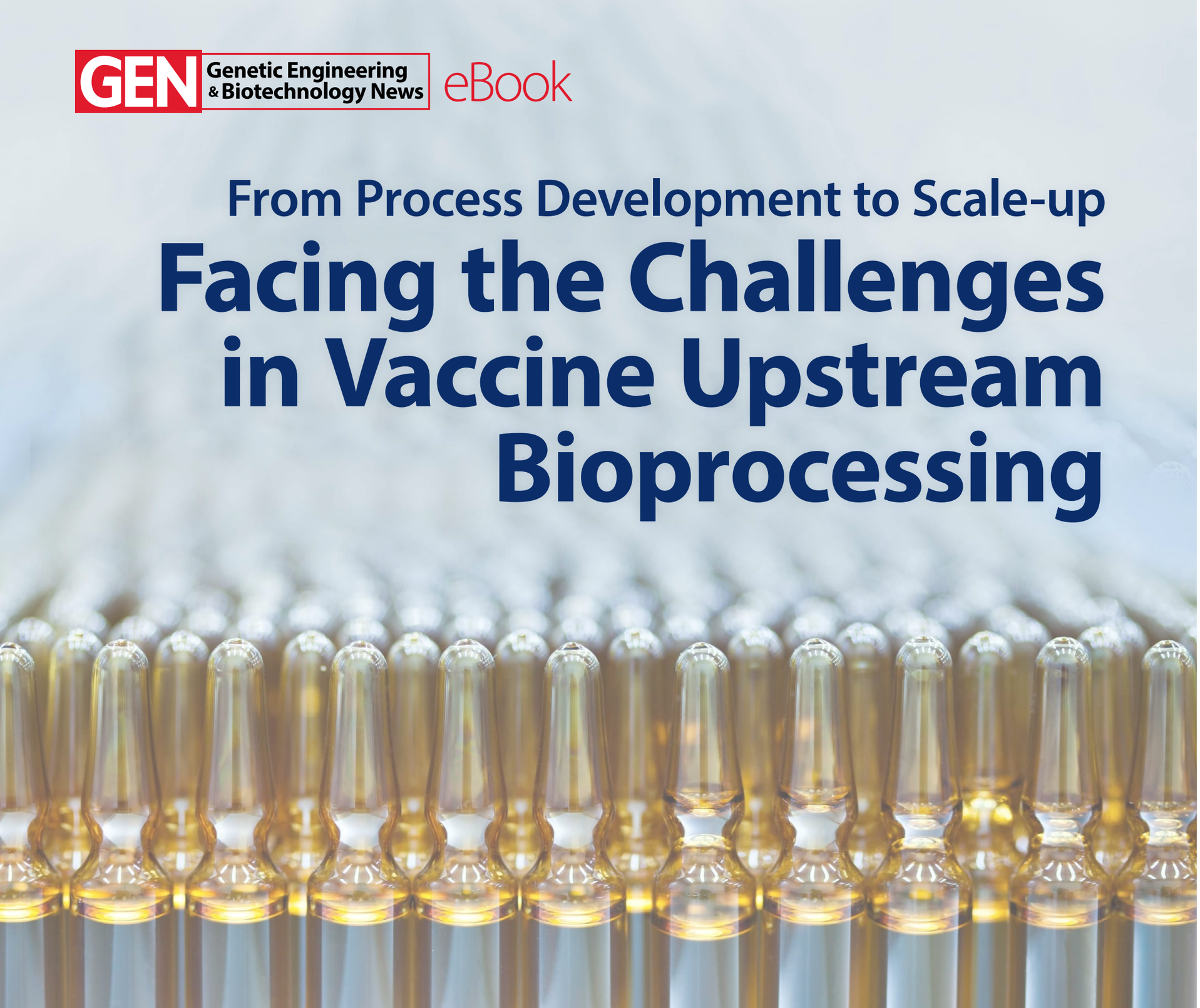
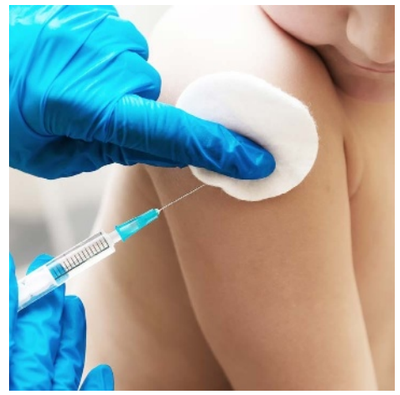
From Process Development to Scale-up

Facing the Challenges in Vaccine Upstream Bioprocessing





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Welcome

Driven by epidemic events and by governmental vaccination programs, there is a rising demand for development of new vaccines and the industry is growing at a double-digit rate. The vaccine industry is facing the challenge of developing new products to serve so far unmet needs and fulfilling the demands on dose numbers, both in an economically viable way.

Upstream bioprocessing is an important piece of the puzzle. High titer, robustness of the process, constant product quality, fast turn-around times, and scalability are some of the success factors. With a comprehensive portfolio of scalable bioreactor and fermentor systems, software, single-use bioreactors, and worldwide service, Eppendorf strives to support bioprocess engineers in tackling these challenges.

With this ebook we would like to share expert views and case studies on some of the hot topics in vaccine bioprocess development. We hope the information on viral vector production, continuous bioprocessing, and process scale-up will prove useful for your own development projects.

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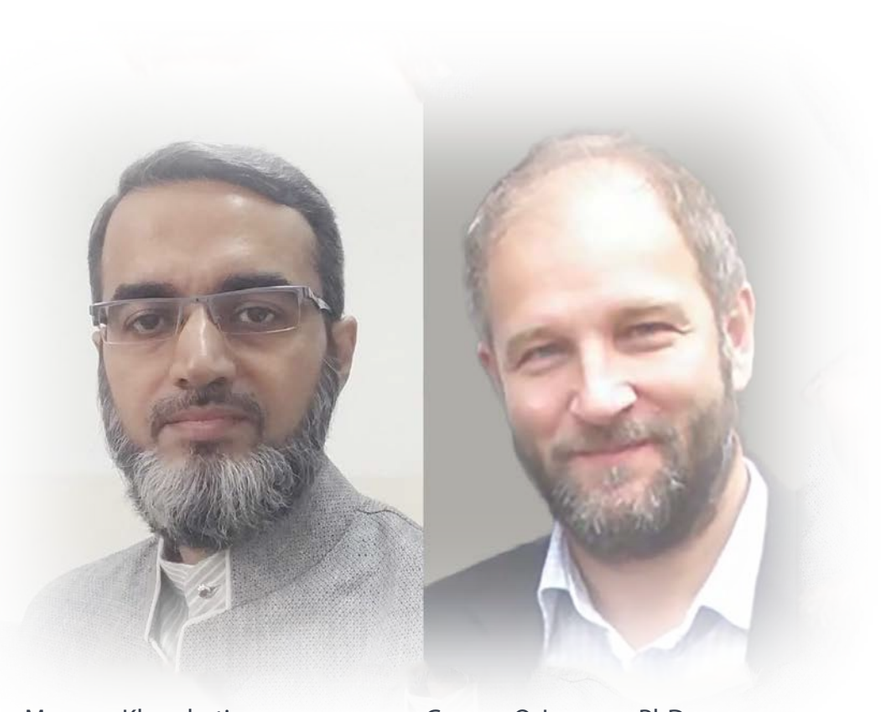

Mannan Khambati | George O. Lovrecz, PhD

The rising prevalence of several diseases and the emergence of several new ones are expected to encourage the development of novel and effective drugs and vaccines, according to a market research study by Transparency Market Research. In 2016, the global vaccines market was worth $28 billion and is projected to reach a value of $48 billion by the end of 2025, notes the study. The market is predicted to register a 6% CAGR between 2017 and 2025.

GEN interviewed Mannan Khambati, of Bharat Serums & Vaccines, India, and George O. Lovrecz, PhD, adjunct professor of RMIT and Monash University, and Senior Principal Research scientist, CSIRO Protein Production and Fermentation, Australia, to get a better sense of the manufacturing and economic issues involved in vaccine development.

GEN: What is currently the most important production platform for vaccines (eggs, cell culture plates, roller bottles, stirred-tank bioreactors)? Do you expect changes in the upcoming years?

Mr. Khambati: On the basis of the general trend and a cost/benefit analysis, a cell culture process utilizing disposable stirred tank or a perfusion system fits perfectly into the scene. As the vaccine industry requires high volume throughput, high yield cell culture along with a perfusion system should be the preferred choice in the upcoming years. Disposable perfusion bioreactor systems should take over from conventional systems to reduce process down-

time, optimize the utility requirement, and reduce equipment footprints, thus leading to compact manufacturing suites and a lower risk of contamination or accidental exposure.

Dr. Lovrecz: Eggs and roller bottles are still the most important platforms, especially for traditional vaccines. This has to change and we all should move to stirred (preferably single-use) tanks. This is made possible by the advances in novel vaccine generation methods (subunits, etc.) and high yield/high density cell cultures.

GEN: Which are the three main challenges in upstream bioprocessing for the production of vaccines?

Mr. Khambati: Low yield process, large volume processing, and high downtime due to maintenance, cleaning, and contamination.

Dr. Lovrecz: Speed and flexibility (in the case of a potential outbreak), and often sterility and operator safety.

GEN: How do you judge the importance of continuous/perfusion bioprocess techniques in vaccine production today? Do you expect it to become more important in the future?

Mr. Khambati: As the global demand for vaccine doses increased, there was a need for a new manufacturing method that could drastically change the way we handle high volumes today. The continuous/perfusion system has come as a savior to vaccine manufacturers as it can resolve both issues of space and volume. With the advent of continuous manufacturing, small footprint equipment with high volume turnaround will become a reality in the near future.

Dr. Lovrecz: Perfusion systems are far more important/central to traditional biologics (mAbs, cytokines, etc.). Yes, I expect it to be become more important in the next five or more years.

GEN: To increase scale we can scaleup (increasing the working volume) or scaleout (increasing the number of bioreactors). Do you think one strategy is more promising than the other?

The continuous/ perfusion system has come as a savior to vaccine manufacturers as it can resolve both issues of space and volume.

Mr. Khambati: In scaleup, usually manufacturing equipment like a bioreactor increases in size vertically while in scaleout the manufacturing suites increase horizontally. Due to constraints in a build area it is always better to scale up than scale out. In regard to the regulatory and operational framework, it is better to have less variability in a process and hence scaleup would be the preferred strategy over scaleout.

Dr. Lovrecz: Scaleout is more important; however, continuous systems and potentially further genetic-engineering manipulations still offer an adequate solution, i.e., we might not have a need for larger single batch volumes after all.

GEN: If you could hand in a wishlist, which improvements in upstream bioprocessing equipment for vaccine process development and production would you like to see?

Mr. Khambati: There are few companies that are manufacturing disposable perfusion systems. I would prefer disposable perfusion bioreactors and continuous purification systems with prefilled resin columns rather than traditional stainless-steel or glass vessels being used in production, as well as in process development. Single-use pump heads, media preparation and storage vessels, sensors, genderless connections, etc. would also be a part of my wishlist.

Dr. Lovrecz: Enhanced biological efficacy (so volumes could be kept low), single-use systems for suspension cells, and improved cell/cell debris removal.

GEN: Do you expect certain cell lines or organisms to lose or gain importance for vaccine production? How important are microbial platforms?

Mr. Khambati: Scientist are studying many unique life forms to harness their potential for use for human health improvement. But microbial platforms are always going to be in demand for vaccine production due to their unique advantages. The enormous database and ease of working with microbes make them preferred choices for most vaccine manufacturing companies.

Dr. Lovrecz: I believe that mammalian cell lines will dominate for another 5–10 years but, potentially, insect lines and yeast systems may gain equal importance.

GEN: For some vaccines, like seasonal flu vaccines, enormous numbers of doses are needed. Which possibilities for the improvement of biomanufacturing do you think will better fulfill these demands and avoid shortages?

Mr. Khambati: With the rise in the number of cases of seasonal ailments like flu, the demand for its treatment is always going to increase. The only way the vaccine industry can fulfill this huge demand is by increasing its manufacturing capacities, improving titers, and harnessing the unlimited potential of continuous manufacturing methods.

Dr. Lovrecz: Better antigenicity (novel platforms such as "Molecular Clamp" or vaccinia virus–based production) and greater yields (culturing conditions/cell lines) should be able to improve the vaccine biomanufacturing process. This might take 3–5 years before we will see significant results.

GEN: Economic considerations are an important factor in vaccine development. Which strategies for cost reduction can you envision to motivate the development of new vaccines to so-far overlooked diseases?

Mr. Khambati: Strategies for making vaccine manufacturing cost effective and viable have always been a big challenge for companies engaged in this business. The application of manufacturing strategies like continous processing, low footprint plant with low utility demands, better process control, and reduced failures can substantially improve the bottom lines. A number of manufacturers are also exploring the possibility of developing mixture vaccines (e.g., pentavalent or hexavalent) as therapies against multiple life-threatening diseases. Thus, reducing the number of dosing events and improving the manufacturing logistics are important economic considerations.

Dr. Lovrecz: Single-use flexible bioreactors using suspension cell lines, better immunogenicity, improved cell lines, higher final density and, perhaps, novel platforms should enable the development of new vaccines especially for the developing world and for overlooked diseases. The Coalition for Epidemic Preparedness Innovations (CEPI) and similar initiatives are reassuring. ■

TUTORIAL

Scalable Production of AAV Vectors

Range of Methods Used in Generating and Purifying AAV Vectors

4X-image/Getty Images

Eva Szarek, Ph.D., and
Jeffrey Hung, Ph.D.

The Vectors derived from adeno-associated virus (AAV) provide promising gene delivery vehicles that can be used effectively in large-scale productions for preclinical target identification/validation studies, or used in large animal models and clinical trials of human gene therapy.

Why is AAV one of the most promising viral gene transfer vectors? Notably, recombinant adeno-associated virus (rAAV) vectors come in different serotypes (AAV 1–9), each with different tissue tropisms. rAAV provides a high rate of gene transfer efficiency, long-term gene expression, and natural replication deficiency. It is nonpathogenic and does not have the capability of altering biological properties upon integration of the host cell.

However, achieving preclinical efficacy testing, especially in large animal models and toxicology studies, requires vector quantities that simply cannot be produced in a laboratory setting or in most research-grade vector core facilities. Current methods for transfection require use of adherent HEK 293 cell cultures, expanded by preparing multiple culture plates. Ideally, a single large-scale suspension culture would be a replacement for multiple culture plates.

In this tutorial, we examine some of the currently available schemes used in generating rAAV from suspension cultures, and describe what it takes to achieve scalable rAAV production.

Scalable Production

Two basic systems for growing cells in culture exist: monolayers on an artificial substrate (i.e., adherent culture) and free-floating in culture medium (i.e., suspension culture). rAAV vector production uses a triple transfection method performed in adherent HEK 293 cells, which is the most common and reliable method (Lock et al., 2010), albeit resource intensive. Due to its scalability and cost, rAAV cell suspensions are more desirable.

To simplify scalability and dramatically decrease operational costs and capital investments, use of bioreactors provides process simplification, from pre-culture to final product. Two examples are the iCELLis from Pall Life Sciences, designed for adherent cell culture applications, and the WAVE Bioreactor from GE Healthcare Life Sciences, ready for batch culture, fed-batch culture, perfusion culture, and cultivation of adherent cells.

Both are designed for convenient handling and control of cell cultures up to 25 L. Both enable rapid and scalable rAAV production. Recently, Grieger et al. (2016) showed suspension HEK293 cell lines generated greater than 1×10^5 vector genome-containing particles (vg)/cell or greater than 1×10^{14} vg/L of cell culture when harvested 48 hours post-transfection, a protocol developed and used to successfully manufacture GMP Phase I clinical AAV vectors.

Large-scale productions require consistent and reproducible. AAV produced for clinical uses must be thoroughly analyzed to identify the main purity, potency, safety, and stability factors described below.

Purity

Empty capsids typically take 50–95% of the total AAV particles generated in cell culture, depending on specific serotypes and protocols used. Empty capsids may solicit deleterious immune response against AAV (Zaiss and Muruve, 2005). It is desirable they be minimized during production and removed during purification.

AAV empty capsids are composed of an AAV capsid shell identical to that of the desired product, but lacking a nucleic acid molecule packaged within. Gradient ultracentrifugation using iodixanol is effective in separating empty capsids. Assessment and measurement can be done by either electron microscopy or A260/A280 spectrometry. It may be difficult to distinguish AAV capsids containing small fragments of DNA

not readily distinguished from completely empty capsids using density centrifugation or electron microscopy.

Helper virus-dependent replication-competent AAV (rcAAV), also referred to as "wild-type" or "pseudo-wild-type" AAV, is an AAV capsid particle containing AAV rep and cap flanked by ITR. This type of AAV (rcAAV) is able to replicate in the presence of a helper virus.

Though wild-type AAV is unable to replicate autonomously and requires co-infection with helper viruses, such as adenovirus, the expression of AAV rep or cap from rcAAV present in an AAV vector increases the risk of immunotoxicity in vector-transduced tissues. Replication competent rcAAV is a rare ($<10^{-8}$) and yet deleterious event.

To assess rcAAV generation, target DNA sequence spans left AAV2 ITR D-Sequence and AAV2 rep sequence. An intact left AAV ITR-rep gene junction is a requisite feature for AAV replication to occur in vivo in the presence of a helper virus. The assay employs sequence-specific PCR primers and a dual-labeled hybridizable probe for detection and quantification of amplified DNA junction sequence.

rcAAV DNA sequence titer is calculated by direct comparison to the fluorescent signal generated from known plasmid dilution bearing the same DNA sequence. A positive signal indicates an intact left IRT-rep gene junction has been detected and amplified, representing the maximum possible rcAAV contamination level present in the rAAV vector sample being analyzed. It does not indicate whether the DNA sequence is infectious or capable of helper-virus assisted replication.

Potency

High potency of AAV vectors is achieved by carefully selecting and isolating full capsids. Physical and functional titers can be measured to assess the actual potency of AAV production. Physical titer measures the encapsidated AAV vector genome, a key mediator and indicator of therapeutic effect. Measurement of vector genomes by quantitative real-time PCR is the closest physical indicator of rAAV vectors. Functional titer is established by measuring transgene protein expression in a dose-dependent manner, following transduction into appropriate cell lines.

Safety

Safety concerns comprise infectious agents used to generate AAV vectors. Mechanisms to inactivate infectious viruses include heat inactivation of adenoviruses and detergent inactivation of enveloped viruses. A complete list of product release tests can include adventitious virus tests of porcine, canine, and bovine viruses (*Table*).

Payload Increase

Developing viral vector comes with key features scientists strive for, including large payload capacity. With rAAV, the limited packaging capacity precludes the design of vectors for the treatment of diseases associated with larger genes; AAV has a packaging capacity of up to 4.5 kb for packaging foreign DNA.

Table. Method of removal and measurement of AAV vector production impurities

AAV Vector Production Impurity	Method of Removal	Method of Measurement
Residual host cell DNA/RNA (nuclease-sensitive)	Nuclease treatment (Benzonase)	qPCR using amplicons to generic host cell genome (e.g., 18SRNA gene); qPCR using amplicons for sequences of specific concern (e.g., AdE1); qPCR using amplicons for non-vector genome sequences
Residual host cell protein	Ultracentrifugation; ion exchange chromatography	ELISA using polyclonal antibodies detecting representative proteins
Residual plasmid DNA (nuclease-sensitive)	Ultracentrifugation; ion exchange chromatography	qPCR using amplicons for helper virus sequences; infectious titer of helper viruses; ELISA or Western blotting for helper virus proteins
Residual helper viruses (nucleic acids and proteins)	Nuclease treatment (Benzonase)	Various, depending on component
AAV empty capsids	Ultracentrifugation; ion exchange chromatography	Electron microscopy; spectrophotometry
Encapsidated host cell nucleic acids (nuclease-resistant)		qPCR using amplicons to generic host cell genome sequences

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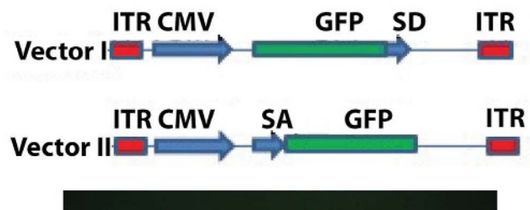

Figure 1. Elements in optimization. Before optimization, the trans-splicing efficiency is about 1–5% when compared to GFP expression from single vector. After optimization, the efficiency reaches about 25–50%.

Table(con't). Method of removal and measurement of AAV vector production impurities

AAV Vector Production Impurity	Method of Removal	Method of Measurement
		qPCR using amplicons to specific sequences of concern (e.g., E1A)
Encapsidated helper component DNA (nuclease-resistant) replication-competent AAV noninfectious AAV particles		qPCR using amplicons for helper backbone sequences
Other, including aggregated, degraded, and oxidized AAV vectors		Ad-dependent amplification; Ad-dependent infectivity in susceptible cells; various, including size-exclusion chromatography, dynamic light scattering, electrophoresis

Utilizing the split vector system, which exploits head-to-tail concatamerization formation, has been developed to circumvent AAV small packaging capacity. Two main approaches include trans-splicing and homologous recombination methods; both depend upon recombination between two vector genomes, with each genome encoding approximately half the transgene, within the same cell to achieve gene expression. ViGene has developed a trans-splicing system that can expand the payload to 8 kb.

We setup a screen for a more efficient trans-splicing system. Here, the GFP reporter was split and cloned into two AAV vectors. Based on GFP expression, after co-transfection in HEK-293 cells, we scored expression efficiency of the combination of different trans-splicing elements and included the selection of a splicing donor and acceptor sequence, and the annealing sequence in the intron. Our best vector generated about 70% expression compared to single vector (*Figure 1*).

We generated and purified both vectors in AAV9 trans-splicing and efficiency and expression levels were tested in vitro and in vivo. Following 72-hours transduction in HEK-293 cells by AAV9 virus *(Figure 2)*, GFP expression was detectable in approximately 70% of cells, about 50% when compared to single vector GFP expression. Similar efficiency and expression levels were observed in RGC neurons, following two-week intraocular injection.

A deeper understanding of the molecular basis for inherited and acquired diseases continues to drive the broader adoption of AAV as the vector of choice for treating many diseases. Numerous Phase I and II trials utilizing AAV have been performed for various inherited and acquired diseases.

A continuation to greatly increase AAV vector yield, improve AAV potency and purity, and increase payload size will further make AAV a bigger player in gene therapy. ■

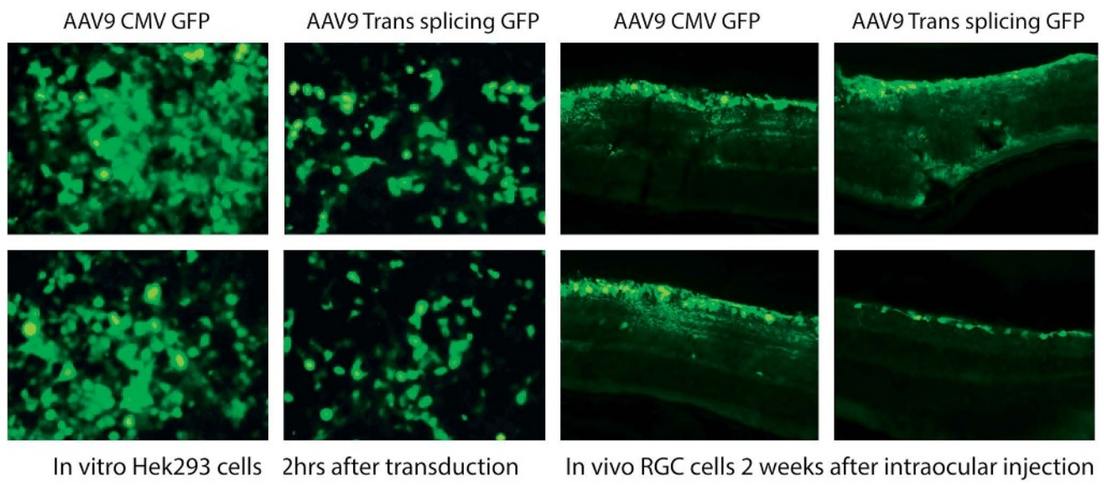

Figure 2. Trans-splicing AAV vector expression of GFP in vitro and in vivo.

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Jeffrey Hung, Ph.D. (jhung@vigenebio.com), is chief commercial officer and GM of cGMP business at ViGene Biosciences.

ROUNDUP

Integrated Continuous Manufacturing of Biologics: Trends in the Field

reptile8488/Getty Images

Biosimilar Manufacturers, Take Note: This Stream Is for You

Randi Hernandez

Although estimates vary slightly, many industry experts predict continuous manufacturing will, at the least, cut the cost of manufacturing biologics in half. Thus, it will be an attractive option for drug makers looking to trim manufacturing budgets. But not all biologics would be feasible candidates for a truly integrated processing stream, and a truly continuous line may require a larger initial capital investment.

To learn more about which companies and products will likely incorporate continuous manufacturing first—and to understand which "hot-button" questions regarding implementation still require attention and clarification—*GEN* spoke to pioneers in the field of continuous biomanufacturing, including Massimo Morbidelli, Ph.D., professor of chemical reaction engineering at the Institute for Chemical and Bioengineering at ETH Zürich; Andrew Zydney,

Ph.D., distinguished professor of chemical engineering, The Pennsylvania State University; Michelle Najera, Ph.D., downstream development scientist, CMC Biologics; Gerard Gach, chief marketing officer, LEWA-Nikkiso America; Dana Pentia, Ph.D., senior application scientist, and John Bonham-Carter, director of upstream sales and business development at Repligen; Gerben Zijlstra, platform marketing manager, continuous biomanufacturing, Sartorius Stedim Biotech; and Karol Lacki, Ph.D., vice president of technology development at Avitide.

Common conclusions from the experts were that the use of surge tanks in continuous lines has both benefits and drawbacks (see the Bioprocessing Perspectives column on page 22 in the September 15 issue of *GEN*); enzyme-replacement products and monoclonal antibodies (mAbs) will likely be the first product candidate types selected for integrated continuous manufacture (more specifically, mAb-based biosimilars); and manufacturers are less likely to switch legacy products from existing batch processes to continuous operation. The high prices of affinity resins are not expected to decline significantly in the near future, and continuous production could result in resin cost savings—but the experts explain there are many benefits of continuous production of biologics besides those related to cost.

Most of the products that would likely be made in integrated continuous lines will be new products, and will also primarily be labile biologics or those which have uncertain demand. Continuous operation, says Bonham-Carter, will allow engineers to react to fluctuations in product demand and give companies the option to build late (or build out) significantly, if required. Dr. Morbidelli asserts that peptides, fusion proteins, scaffolds with mAbs, and other products "containing sensitive antennary glycostructures that are needed for biological activity" would also be types of therapies that could be made in continuous flows.
To tell which pharma manufacturers are likely to integrate end-to-end continuous manufacture into production lines first, investors and industry insiders should follow company patent filings and peer-review articles authored by company representatives. As Dr. Zydney points out, manufacturers that are already "clearly interested" in fully continuous biomanufacturing lines include Genzyme, Merck, Bayer, and Sanofi, among others. Also doing work in this space: Novo Nordisk, Novartis, Amgen, Shire, Pfizer, WuXi, and BiosanaPharma. In addition, some contract manufacturing organizations (CMOs) are in the continuous biomanufacturing field (such as CMC Biologics), and these organizations are poised to help biopharma clients lower the cost to clinic by way of fully continuous manufacturing strategies.

Regardless of who is or is not investigating continuous, "What is definitely a right development trend is that vendors do support the change and offer technologies that enable continuous operations," says Dr. Lacki of Avitide, a company that makes affinity resins. "At the end of the day, it will be up to the end user to decide how a process needs to be operated, but the choice will be made on a thorough assessment of commercially available technologies."

GEN: What types of biologic medications are the best/most feasible candidates for integrated continuous manufacture?

Dr. Najera: Any product with an expensive chromatography resin is a suitable candidate for continuous chromatography, especially for early-phase clinical products, where resin cost can be cost prohibitive. Continuous capture is also attractive for high-titer processes, since several small volumes can be used instead of a single large column. In fact, facility fit limitations are common for high-titer processes (>5 g/L), and continuous chromatography can help manufacturers avoid the capital costs associated with procurement of large stainless-steel columns (>80 cm diameter) and associated equipment. Finally, the concept of integrated continuous manufacture, (i.e., a fully continuous process), would be best suited for a well-established late-phase process with a stable market demand where cost-savings are realized through basic efficiencies related to batch versus continuous processing.

Dr. Zydney: This is a difficult question to answer—it very much depends upon one's perspective. In some ways, the 'best' candidates

Integrated Continuous Manufacturing of Biologics

Karl Rix, Ph.D.,
Head of Business Unit Bioprocess, Eppendorf

GEN: What types of biologic medications are the best/most feasible candidates for integrated continuous manufacture?

Dr. Rix: Besides for the production of less stable proteins which in a perfusion process are constantly harvested from the culture, perfusion processes could be especially advantagous to respond to fluctuations in the market. Scale can be varied by modulating the process time or by changing the number of parallel production lines. This can be easier than traditional scale-up of a fed-batch process.

GEN: The manufacturers of Prezista (Janssen), a small-molecule drug, got FDA approval to change its processes to a continuous method. To your knowledge, are any biologics manufacturers looking into a similar manufacturing change? Do you think manufacturers are more likely to address the end-to-end manufacture of a totally new product, rather than for an existing product?

Dr. Rix: Though there have been examples, it is still quite unlikely that a manufacturer would change an approved batch or fed-batch manufacturing process to continous operation. Usually, the decision for a process mode will be made in product development. In that phase, influencing variables, including product titer, costs for media, labor, and equipment, and product quality, can be systematically assessed.

GEN: To date, why do you think so few biologic manufacturers have explored the use of an end-to-end continuous line?

Dr. Rix: In my view, one issue ist that process development as well as process monitoring strategies for continuous processes are less well established than for traditional fed-batch processes. This includes among others cell line and media development, online monitoring technologies, and scale-down model development. This might increase process development time and risks. And in my opinion, regulatory uncertainties are still an issue.

for integrated continuous manufacturing are products for which there are particular challenges in using batch operations. For example, a highly labile product that degrades over time would benefit dramatically from the use of a continuous process—this is why perfusion bioreactors were originally used for production of unstable clotting factors. On the other hand, integrated continuous manufacturing is well suited for products that have very high-volume demand or significant cost constraints. The successful development of integrated manufacturing systems will likely require considerable product and process knowledge, which today is most readily available for mAb products due to the large number of these products already in commercial manufacture.

Dr. Lacki: I would rather ask a different question: What type of expression systems or upstream technologies are more suited for continuous operation? And my answer would be perfusion operation or even [use of] six-pack fed-batch bioreactors would make an operation quasi-continuous. That said, I think that a successful continuous biomanufacturing process must be as simple, or rather, as robust, as possible. For instance, a downstream process that relies on as few chromatography steps as possible will be more suited for continuous operations. From that perspective, one could argue that an affinity step is an enabler for continuous downstream operation. Cost of an affinity resin will be lower if it is operated in a continuous manner, but even without that, the benefit of normalizing a product stream through a selective capture step would deliver so many advantages from the process reproducibility and controllability perspective that the cost of the resin need not even be considered.

Mr. Zijlstra: I would say [the most feasible type of therapies would be] primarily labile products that require perfusion and immediate capture from the cell broth to prevent product degradation. Some cost-model studies also suggest that beyond certain annual production scales, integrated continuous biomanufacturing leads to lower COGs for mAbs and are preferable in that case. Finally, evidence is mounting that even for mAbs, (critical) product quality attributes can be controlled much more tightly [in continuous than] in batch operational mode.

GEN: The manufacturers of Prezista (Janssen), a small-molecule drug, got FDA approval to change its processes to a continuous method. To your knowledge, are any biologics manufacturers looking into a similar manufacturing change? Do you think manufacturers are more likely to address the end-to-end manufacture of a totally new product, rather than for an existing product?

Dr. Najera: While it is easy to understand why people draw parallels between small molecules and large molecules, it is important to note that control of small vs. large molecules is significantly different. For small molecules, it is relatively easy to understand all impurities and variants through analytical testing. This is not always possible for large molecules. For this reason, continuous processing and process analytical testing (PAT) are often linked. Even with the challenges large molecules pose, there are many companies presenting compelling small-scale data on continuous purification strategies. However, these processes are

inherently more complex and are perceived to pose higher risk. To the question, it is unlikely that an existing commercial manufacturing process would shift to a continuous process unless there was an extremely compelling cost benefit in doing so. The benefits would have to offset the cost of process development, establishing a new process at-scale, process validation, and potentially, new clinical studies. Additionally, significant facility modifications would likely be needed to convert a fed-batch facility to a continuous facility. For this reason, it is more likely that a fully continuous process would be developed for a new product.

Dr. Lacki: A change of a legacy process to a continuous version will only happen if a second- [or] third-generation of a process is being considered anyway. However, change of the infrastructure might prove financially prohibitive. A different story is if a company is building a new facility for the legacy product or for a brand-new API; in that case, continuous operation will definitely be evaluated as the capital investment associated with a continuous plant, at least from the equipment-side perspective [and] should be much lower compared with batch-based manufacturing.

Dr. Zydney: I am not aware of any biomanufacturer that is currently looking to replace an existing batch process with an end-to-end continuous process. I think it is more likely that a fully continuous process will be first developed for a new product that is particularly well-suited to continuous manufacturing, or potentially for a biosimilar where the cost reduction would be particularly attractive.

Dr. Morbidelli: It is now demonstrated that continuous processes provide higher quality and more homogeneous products, which are beneficial for the patient. This justifies the strong support that FDA always provided to the development of these new manufacturing technologies. In addition, these can also lower the production costs in the broad sense, which will obviously impact the growing market of biosimilars. This is why all major pharma companies are looking carefully at developments [in continuous manufacturing], although I do not know which approach they will select to implement this important transition.

Mr. Bonham-Carter: No biopharma would consider changing an existing commercial manufacturing process, in my view. Some might consider changes between Phase II and Phase III, for reasons of capital risk, uncertain market demand, tighter quality requirements, or manufacturing network management.

GEN: What are the financial implications of a truly continuous biomanufacturing line?

Mr. Bonham-Carter: Estimates range up to >80% cheaper in capital costs, but perhaps only 20–60% in cost of goods. Most models are relatively simple and do not take account of a company's portfolio of drugs, risks of failure at different phases, quality requirements for different drugs, and existing manufacturing and engineering skill—each of which will impact true cost to the manufacturer.

Dr. Morbidelli: It depends which parts of

the process are exchanged to become fully continuous, and those which would benefit from intrinsic step process improvements. A conventional fed-batch process can be nicely coupled to continuous downstream purification with one of several steps performing continuous batch (flip-flop) or countercurrent processes (capture and polish). If combined with membranes and single-use concepts, we expect a significant productivity increase having an implication on both CAPEX and OPEX. We estimate that besides the improvement in product quality, both CAPEX and OPEX could be lowered by 50%.

Mr. Zijlstra: Continuous and single production plants will be able to lower costs several fold and produce the same amount of product as current standard stainless-steel facilities. This facilitates greenfield investment decisions and reduces investment risks considerably.

Dr. Lacki: A standard argument is that equipment for a continuous line will be smaller. At the same time, truly continuous operations run 24/7, which means that even the downstream operation might need to be run in shifts, increasing labor cost. Undoubtedly, continuous operation will be more control-heavy. Cost of controllers, equipment maintenance, contingency plans—all of it needs to be considered when evaluating continuous processes. That said, advances in detection technologies and modularization of standard processing technologies (e.g., prepacked columns, flow paths, and even introduction of humanoid robots) will bring us closer toward continuous processing.

GEN: To date, why do you think so few biologic manufacturers have explored the use of an end-to-end continuous line?

Dr. Morbidelli: We think there are actually quite a few [therapies] in the exploration phase for either perfusion culture or continuous downstream or both. We know that manufacturers are currently exploring continuous systems at the pilot scale [that] would move into clinical trial manufacturing in 2018. FDA has consistently supported and guided continuous manufacturing and we believe that the regulatory risk for a new product development is low. As with any new technology, continuous manufacturing for biologics will need some evaluation time to be taken into the commercial setting, as the technology has to show that it is robust and provides the expected benefits. There might be a level of confusion as to the quality of available technology platforms with different claims on performance/productivity. We believe that a strict scientific approach in evaluating the benefits of each technology may drive the choice to platforms that are simple and robust, reducing the validation effort and likeliness of hardware failure in continuous operation.

Dr. Lacki: The short answer could be, a lack of real need. The industry is still young, and fairly profitable. The focus from the beginning was more on drug safety than on reducing the cost of goods. But as in the case of all industries, with technology maturation comes challenges related to implementation of technological solutions that will be able to address the market pressure, without sacrificing the safety profile of a product. The pressure is both related to the pure amount that needs to be produced (number of medicines

keeps increasing) and to the expectation from society to make these medicines affordable for all. Personally, I don't think the [biologics] industry will ever approach the commodity industry where every tenth of a cent counts—but it will definitely aspire to lower the manufacturing costs, to deliver affordable medicines, and to stay competitive against its peers. The patent cliff is here to stay.

Mr. Zijlstra: Many large pharma companies are actively testing intensified and continuous approaches in their advanced research and development labs. Intensified technologies are being implemented as we speak. However, before the fully continuous technology is accepted by manufacturing as a replacement for the existing, proven, and robust platforms, there is still some technological and regulatory strategy development required.

Dr. Najera: I think it is related to a struggle within the industry to be able to define a single batch or a lot of drug product from a regulatory perspective. A drug product is not complete without the associated paperwork that describes the processing (e.g., batch records, analytical results). Continuous manufacturing really challenges us to rethink how to present this paperwork to sufficiently demonstrate how the purification process to make that drug is under control. For example, a continuous process may rely on time-based process analytical data showing that the product quality is well controlled, rather than analytical results for intermediates at specified points in the process. In sum, although the regulatory agencies have encouraged the introduction of more continuous processes, a strategy for how to define a continuous batch' it not straightforward.

Mr. Gach: The 'continuous' market is in a stage like when single-use was first introduced—it was adopted in the areas where [it would have] the highest benefit or were the most developed areas, and only recently has grown into a full-on production scenario. Presently, leading companies are actively applying continuous technology in unit operations where they are feeling the most pain and the technology is straightforward. With these experiences, they will gain confidence and (regulatory) buy-in to expand further up- or downstream. Some will eventually grow into full continuous, while others will gain significant benefit using 'continuous' technologies to debottleneck batch operations.

Dr. Zydney: Biomanufacturing tends be conservative' in its approach—the primary goal is to provide a manufacturing environment that is capable of delivering the desired biotherapeutic in a fully robust fashion, while satisfying all safety and regulatory requirements. Fully continuous biomanufacturing is currently untested'; there is inherently greater uncertainty regarding the manufacturing process, including the approval of that process by the appropriate regulatory agencies. Many biopharmaceutical manufacturers would be happy to be second' in the development of a continuous process, but they are very reluctant to be first. Some of this is cultural/organizational—the individuals involved in the development of new technology solutions that will be needed in continuous bioprocessing may not be the people making the final decision on the design of the manufacturing facility. However, considerable progress is being made, both by

technology providers and biomanufacturers—I think it is only a matter of time before we see a continuous process implemented at pilot scale (e.g., for production of a biotherapeutic for use in clinical trials), and then ultimately, at manufacturing scale.

Dr. Pentia: It is still early [for many companies to consider] continuous biomanufacturing. The idea only started to be taken seriously approximately five years ago. The industry is still trying to understand where it is appropriate and how to fully control continuous manufacturing at large scale (tens of products are already produced in perfusion at commercial scale). I would not say there are too few manufacturers that are exploring this—it might be more accurate to say that every manufacturer is considering this approach, but some are further advanced than others, while others are just in the conceptual phase. ■

WHITE PAPER

rAAV Production in Suspension CAP GT® Cells in BioBLU® 3c and 10c Single-Use Vessels

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Abstract

In pharmaceutical and biotech industry there is an increasing interest in gene therapy-based applications. This leads to an increasing demand in scalable production systems for viral vectors. Cell culture in suspension instead of monolayer culture simplifies scale-up, because it allows bioprocessing in scalable, stirred-tank bioreactors. Researchers at Cevec® Pharmaceuticals adapted a small scale shake flasks rAAV production process to stirred-tank bioreactors and scaled it up in BioBLU Single-Use Vessels from a working volume of 2 L to 10 L. Scale-up in bioreactors was based on constant power input/volume. It led to comparable cell growth and virus production at both scales and to the original shake flask process.

Introduction

Bioprocess development is usually carried out at small working volumes. This helps saving time and resources, because at small scale several

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experiments can be conducted in parallel, costs for media are kept low, and not much lab space is required to operate small-scale bioreactors. But in the course of biopharmaceutical development more material is needed for characterization, trial runs, and finally for commercialization. To maintain product yield and product quality while scaling up, bioprocess engineers usually aim to keep one or more process parameters constant across scales.

Stirred-tank bioreactors with similar vessel geometries and capabilities across scales simplify scale-up, as they allow keeping critical parameters constant. Parameters to describe the vessel geometry include impeller diameter, vessel diameter, liquid height and ratios thereof. Key engineering parameters related to scale-up include the tip speed, mixing time, volumetric mass transfer coefficient (kLa), and the power input/volume ratio (P/V). It depends on the process, which of these parameters is most important. Cevec Pharmaceuticals uses a unique human cell–based expression system (CAP Technology) in two product portfolios. One is the glyco-optimized CAP Go® cell line for tailor-made production of N- or O-glycosylated

Figure 1. BioFlo 320 bioprocess control station (left) and the BioBLU c Single-Use Vessels (right) used in this study.

proteins, the other is the CAP GT cell platform for stable and transient industrial-scale production of recombinant adeno-associated viruses (rAAV), lentiviral, and adenoviral gene therapy vectors. In this study, researchers at Cevec aimed at scalingup a rAAV transient production process using CAP GT cells. When scaling up, they maintained constant P/V between vessels, which is one of the most prevalent strategies for scale-up.

Material and Methods

Cell line and medium

Human CAP GT cells were cultivated in a chemically defined, animal component-free medium compatible with transient transfection.

The thawing, seedtrain and amplification of the CAP GT cells was performed in shake flasks in suspension, to generate sufficient biomass for seeding the final production vessel (stirred-tank bioreactor). The cell culture in the bioreactor was inoculated to an initial cell density of 5 x 10^5 cells/mL.

Bioprocess system and process parameters

The bioprocess engineers at Cevec used a BioFlo 320 bioprocess control station equipped with BioBLU 3c or 10c Single-Use Vessels *(Figure 1)*. BioBLU Single-Use Vessels have an industrial, rigid-wall design. They provide fast and efficient mixing with magnetically coupled overhead drives.

The vessel geometry is similar across scales, among others in terms of the ratio of impeller diameter to vessel inner diameter and the ratio of maximum liquid height to vessel inner diameter. This makes it easy to use the BioBLU vessels at small scale during process development and then scale up the process to larger working volumes. The researchers set the temperature to 37 °C. The pH was regulated with CO_2 (acid) and sodium bicarbonate (base). The vessels were equipped with a macrosparger and one pitched-blade impeller. The gassing strategy was an automatic gas mix, which automatically controls air, oxygen, nitrogen, and CO_2, depending on the pH and dissolved oxygen (DO) set points. The agitation speed of the BioBLU 3c vessel with a 2 L working volume was set to 200 rpm (corresponding to a tip speed of 0.69 m/s); for the BioBLU 10c with a 10 L working volume, it was 175 rpm (corresponding to a tip speed of 0.84 m/s).

Virus production process

The bioprocess for rAAV production was divided into four phases.

1. Expansion phase: CAP GT cells were expanded in suspension in BioBLU Single-Use Vessels.

2. Transient transfection: At 72 hours into the process, cells were transiently transfected with a two-plasmid system from PlasmidFactory® encoding for rAAV-GFP. Transfection was mediated by Polyethylenimine (PEI). With the transfection the production phase was started.

3. Production phase: During this phase the cells produced rAAV-GFP.

4. Harvest phase: The culture medium was collected and processed to harvest the virus particles. Phase 4 is not further described in this application note.

Power number

The impeller power number is a dimensionless number associated with different types of impellers. The impeller power number is used to calculate P/V using the following equation:

$$P/V = (Np \times \rho \times N^3 \times d^5)/V$$

with Np being the impeller power number, ρ being the density of water (1000 kg/m^3), N being the agitation speed (rps), d being the impeller outer diameter (m), and V being the full working volume (m^3). To calculate P/V, the bioprocess engineers at Cevec used impeller power numbers which were experimentally determined in the Eppendorf applications laboratory. Experimental determination of power numbers using a rotational torque sensor requires a direct drive motor. To be able to measure the power numbers for BioBLU Single-Use Vessels—which have a magnetic drive—, the Eppendorf application scientists modified the vessels to remove the magnet from the magnetic drive coupling. They then connected the torque sensor directly to the impeller shaft.

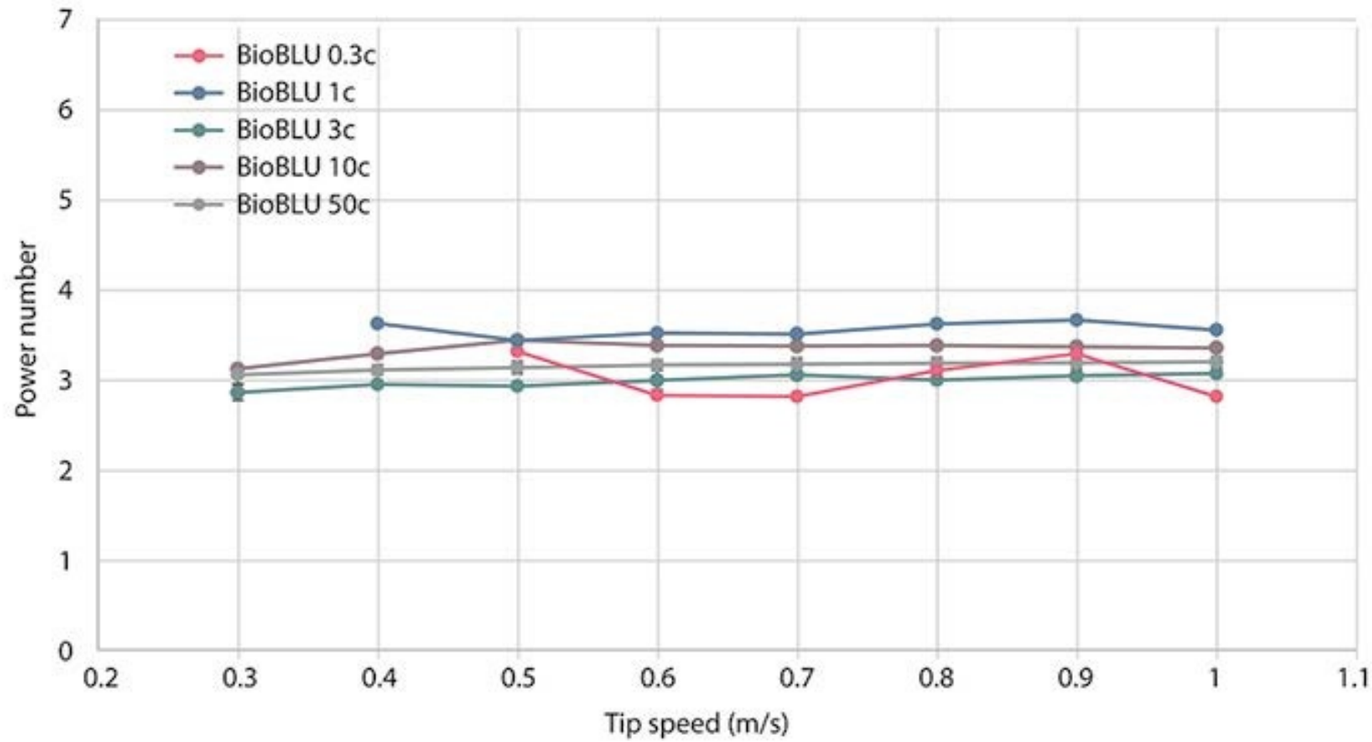

Figure 2. Power numbers of BioBLU c Single-Use Vessels with one impeller were determined according to [1] and [2].

Power numbers were measured at tip speeds between 0.3 and 1 m/s and then the average was calculated. For the BioBLU 3c the mean was 2.98 and for the BioBLU 10c the mean was 3.33 *(Figure 2)*.

Scale-up strategy

The scientists optimized the agitation speed during process development. Scale-up was based on similar power input at both scales. The agitation speed of the BioBLU 3c vessel with a 2 L working volume was set to 200 rpm (corresponding to a tip speed of 0.69 m/s); for the BioBLU 10c with a 10 L working volume, it was 175 rpm (corresponding to a tip speed of 0.84 m/s). This results in comparable power inputs of around 62 W/m^3 at both scales.

Analytics

The bioprocess engineers determined the viable cell density and cell viability offline using a NucleoCounter® NC-3000™ (ChemoMetec®, Denmark). They analyzed the transfection efficiency by measuring GFP fluorescence with a NucleoCounter NC-3000. Productivity was measured by quantification of the viral genome titer by qPCR.

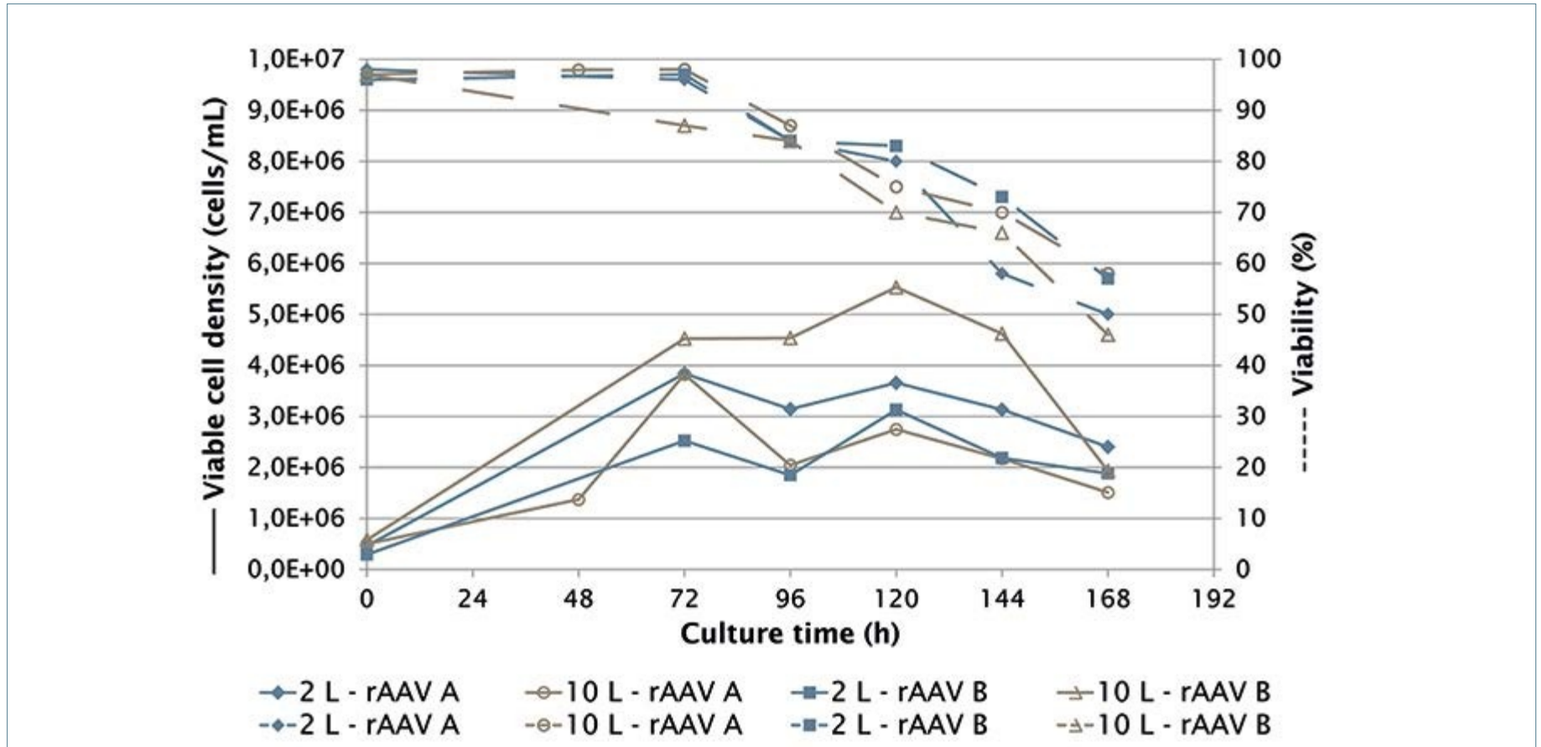

Figure 3. Cell growth and viability.

Results

The researchers compared the performance of the initial shake-flask process with bioprocess performance at working volumes of 2 L and 10 L. They conducted the same production process in two duplicate runs runs at the 2 L scale (2 L rAAV A and rAAV B) and two duplicate runs at the 10 L scale (10 L rAAV A and rAAV B). For the two duplicate runs at 2 L and 10 L, the bioprocess engineers plotted the viable cell density and viability over time *(Figure 3)*. Similar growth patterns were achieved. The viable cell density at the point of transfection was similar to the original shake flask process (data not shown). Post transfection at 72 h, viability dropped in a similar manner in all runs. This was expected, as virus production is cytotoxic. The DO control was very tight at both scales,

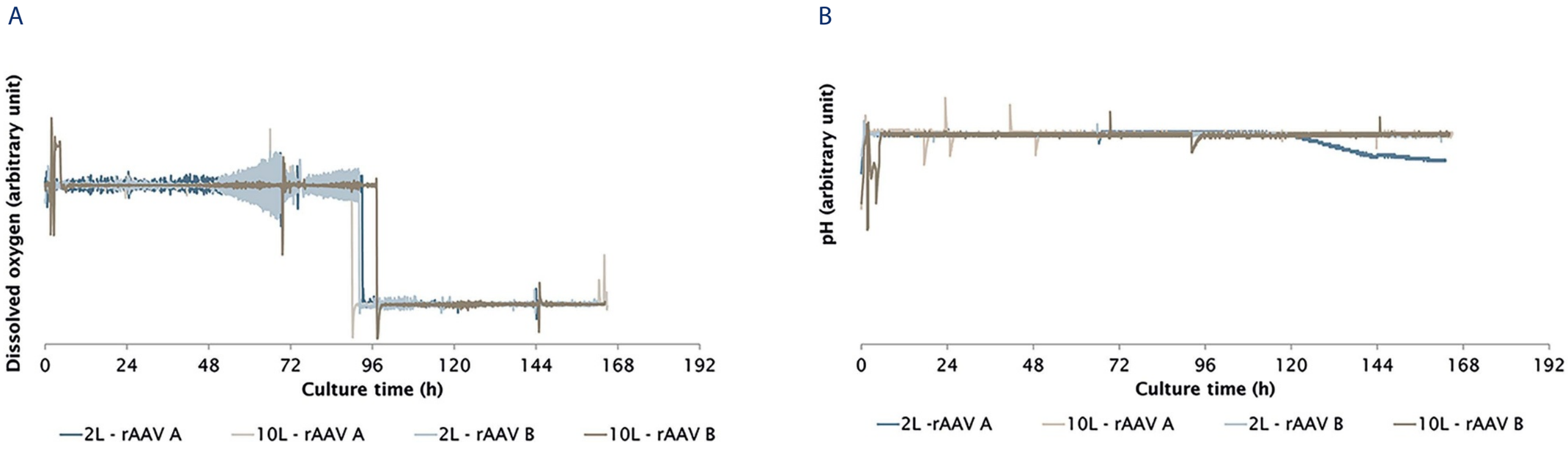

Figure 4. A. DO control. B. pH control.

with similar profiles for all four runs *(Figure 4A)*. All four runs showed similar pH profiles within the regulation parameters *(Figure 4B)*. The researchers determined transfection efficiency based on green fluorescent protein (GFP) fluorescence measured with a NucleoCounter NC-3000. Similar transfection efficiencies of around 70 % were achieved at both scales. The transfection efficiency was comparable to that of the original shake flask process *(Figure 5)*.

The bioprocess engineers at Cevec measured productivity by quantification of viral genome titer by qPCR. The viral genome titer was similar in the bioprocess runs with a working volume of 2 L and 10 L and again comparable to the original shake flask process *(Figure 6)*.

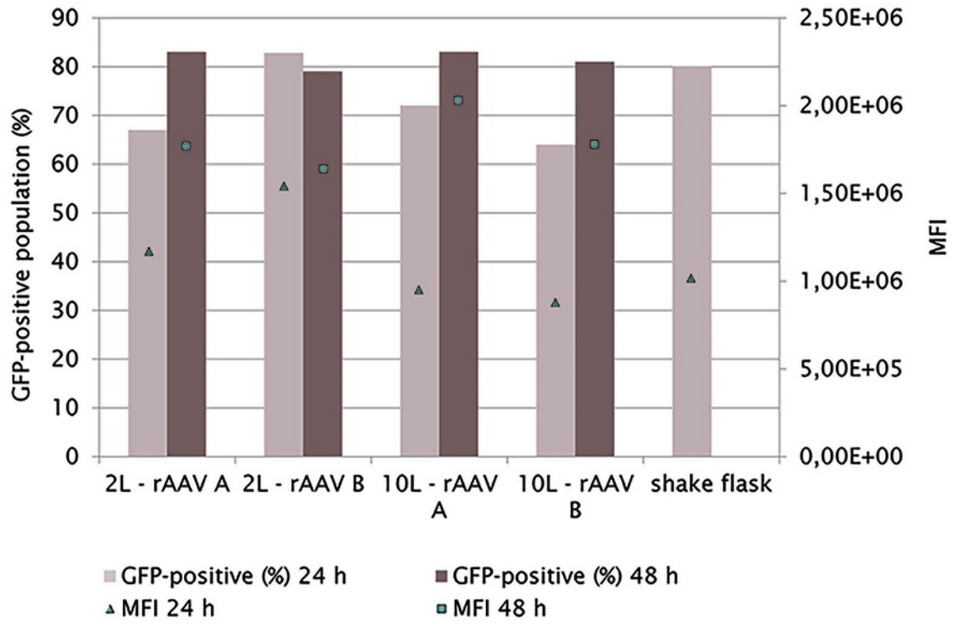

Figure 5. Transfection efficiency

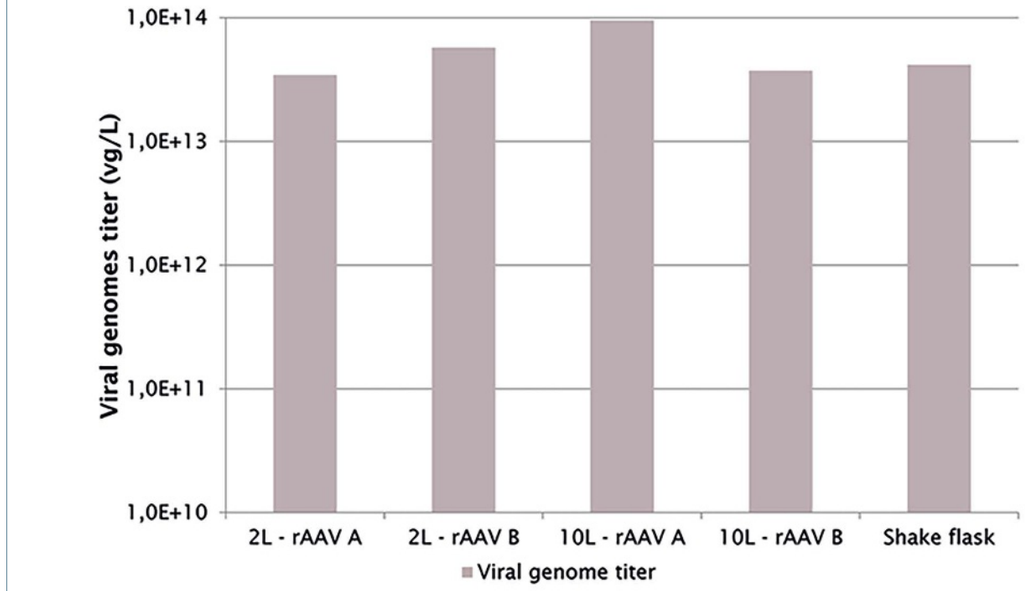

Figure 6. Productivity

Conclusion

Bioprocess engineers at Cevec scaled-up a rAAV production process from BioBLU 3c Single-Use Vessels with a working volume of 2 L to BioBLU 10c Single-Use Vessels with a working volume of 10 L. Cell growth, transfection efficiency, and productivity were comparable at both scales. This process development enables then the production of higher amounts of rAAV gene therapy vectors by transient transfection. Moreover, these results give an example for successful bioprocess scale-up based on constant P/V using differently sized BioBLU Single-Use Vessels. ■

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Vero Perfusion, Packed-Bed Vessels Intensify Vaccine Production

Eppendorf's Fibra-Cel and BioBLU 5p Technologies Show Promise for High-Titer Vaccine Production

Art Marie/Getty Images

Xiaofeng (Kevin) Han, Ph.D.,
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and Ma Sha, Ph.D.

Strong demand for vaccines for viral diseases requires that manufacturing techniques become more productive, including those based on scalable bioreactor cell culture systems. In viral vaccine production, Vero is one of the most widely used cell lines.[1] Because these cells are anchorage dependent, they require a growth support when they are cultivated in stirred-tank bioreactors. For high-density Vero cell culture processes, an attachment matrix incorporating Fibra-Cel® disks shows promise.

The disks are made of polyester and polypropylene fibers, which provide a three-dimensional environment, offering a high surface-to-volume ratio and protecting cells from damaging shear forces. In combination with a basket impeller, a packed-bed bioreactor system is formed, which enables the exchange of growth medium and harvest of the end product in perfusion processes, without the need for cell filtration.

Figure 1. BioBLU 5p Single-Use Vessel

In this study, we cultivated Vero cells in perfusion mode in Eppendorf BioBLU® 5p Single-Use Vessels with a built-in basket impeller prepacked with Fibra-Cel (*Figure 1*). We achieved a high cell density of approximately 43 million cells/mL, demonstrating great potential for Vero cell–based vaccine production using Fibra-Cel packed-bed vessels.

Materials and Methods

Cell Line and Medium

We cultivated adherent Vero cells (ATCC, CCL-81TM) in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific), supplemented with 1× Antibiotic-Antimycotic (Thermo Fisher Scientific) and 1% (v/v) heat-inactivated fetal bovine serum.

Growth Surface

We cultivated Vero cells in a BioBLU 5p Single-Use Vessel with a working volume of 3.5 L. Each BioBLU 5p contains 150 g of Fibra-Cel disks. These provide a growth surface of 180,000 cm^2, which is equivalent to about 212 roller bottles, or 29 10-layer, stacked-plate, cell culture vessels.[2]

Bioprocess Control

The bioprocess was controlled with a BioFlo® 320 bioprocess control station. The BioBLU 5p Single-Use Vessel was inoculated with 530 mL of inoculum, which had a cell density of 3.0×10^6 cells/mL and a cell viability of 99%, resulting in an inoculation density of 4.6×10^5 cells/mL. The cells were cultivated at 37°C.

Dissolved oxygen (DO) was measured using a polarographic ISM® sensor (Mettler Toledo). DO was controlled at 50% by automatic gassing at a flow of 0.002 – 0.5 SLPM in the 3-Gas Auto mode via a macrosparger. To reduce foaming caused by high gas flow in the late culture stage, we limited air flow to 0.002–0.2 SLPM and oxygen flow to 0–0.5 SLPM.

The BioBLU 5p Single-Use Vessel was equipped with an optical pH sensor and the pH was controlled at 7.1 (deadband = 0.1) via a cascade

of C_{O2} (acid) and 0.45 M sodium bicarbonate (base). The culture was agitated at 100 rpm. We added Antifoam C Emulsion (Sigma-Aldrich) when it was needed.

Feeding and Perfusion Control

We started with a perfusion rate of 0.2 vessel volumes per day (VVD) and gradually increased it to 1.5 VVD at the end of the run. We determined the perfusion rate by monitoring metabolite levels with a Cedex® Bio Analyzer (Roche Diagnostics). The goal was to keep the ammonium concentration below 4 mM and the glucose concentration between 2 and 4 g/L.

In addition to perfusion, we performed extra glucose bolus feeding (200 g/L glucose stock solution) based on the glucose level in the bioreactor at the end of every day. The aim was to bring the glucose level in the bioreactor close to the glucose concentration in the perfusion medium (4.7 g/L) at the beginning of the next day. We also determined lactate and glutamine levels daily.

Cell Growth

In the packed-bed bioreactor, the Vero cells cannot be counted directly during the culture because the cells adhere to the Fibra-Cel disks. To obtain an indirect measure of cell growth, we analyzed the glucose consumption of the culture.

The rate of glucose consumption (R) in grams per day can be calculated based on the total glucose added to the bioreactor minus the residual glucose. The daily total amount of glucose added to the bioreactor is therefore equal to the amount of glucose in the vessel at the start of the day ($G_{\text{vessel-start}}$) combined with the glucose supplied through perfusion ($G_{\text{perfusion}}$) and the extra glucose added via bolus feed (G_{bolus}). By subtracting the amount of glucose remaining in the vessel at the end of the day ($G_{\text{vessel-end}}$) as well as the amount of glucose remaining in the harvested perfusate (G_{harvest}), we arrive at the amount of glucose consumed (g) per day (24 h). The daily glucose consumption rate is represented by the following equation:

$$R = (G_{\text{vessel-start}} + G_{\text{perfusion}} + G_{\text{bolus}} - G_{\text{vessel-end}} - G_{\text{harvest}}) \text{ / day}$$

The glucose amount in the vessel at the start and the end of the day can be calculated by multiplying the glucose concentration in the medium by the working volume. The amount of glucose added by bolus feed can be calculated based on the concentration of the glucose stock solution and the volume of the bolus feed. The amount of glucose supplied through perfusion can be calculated based on the glucose concentration in the perfusion medium and the perfusion volume. The amount of glucose remaining in the harvested perfusate can be calculated based on its volume. In addition, we directly measured the cell number at the end of the cell culture run by counting nuclei using a crystal violet nuclei counting assay (Chemglass Life Sciences, CLS-1332-01). We cut the vessel open below the head plate and collected Fibra-Cel samples from two different locations in the basket. We extracted the cell nuclei, stained them with crystal violet, and counted them using a Vi-CELL® XR Cell Viability Analyzer (Beckman Coulter) as described previously.[2]

Results

Confirming Cell Attachment

To assess Vero cell attachment on the Fibra-Cel disks, we took a culture sample 30 minutes after inoculation. No cells were observed in suspension, indicating that the Vero cells had attached to the Fibra-Cel disks rapidly. We started the perfusion on day 3, because the glucose level became low and the concentration of the toxic byproducts lactate and ammonium increased.

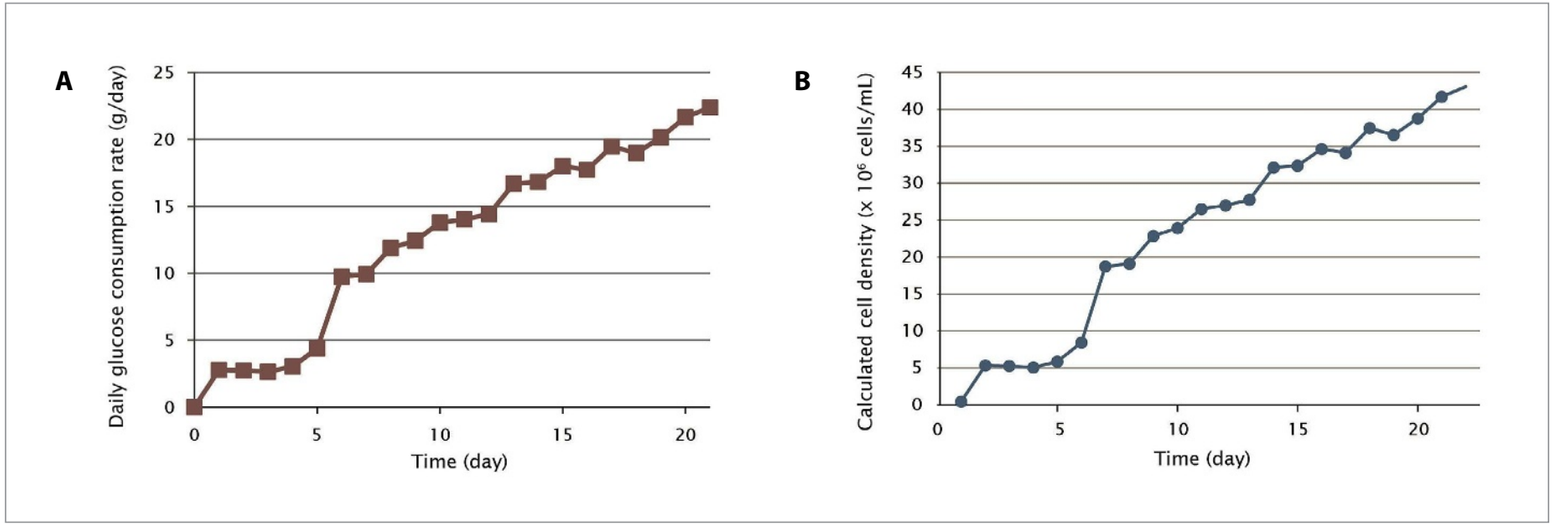

Figure 2. (A) Daily glucose consumption rate of the culture. (B) Calculated Vero cell growth curve.

Tracking Glucose Consumption

Using an indirect measure of cell growth, we tracked the glucose consumption rate of the culture (*Figure 2A*). We calculated the total glucose consumption of the culture and the daily glucose consumption rate.

For the first four days, the daily glucose consumption rates were around 2.7 g/day, indicating that cells were in lag phase. From day 5, the glucose consumption rate started to increase to 4.4 g/day, and even doubled to 9.7 g/day on day 6, indicating that cell growth had entered the log phase. Cells were growing continuously, and the glucose consumption rate doubled again to 19.4 g/day on day 17. On the last day of the run, the glucose consumption rate was still increasing, reaching 22.4 g/day. In total, the cells consumed 273 g glucose during the 21 days.

Calculating Vero Cell Growth Curve

At the end of the process, we determined the cell number by counting nuclei. For one Fibra-Cel disk sample, the average cell count was 4.43×10^6 cells. From this number, we extrapolated the total number of cells in the culture as described previously.[2] As a result, the total number of Vero cells in the BioBLU 5p vessel at the end of the perfusion culture was calculated as 1.51×10^{11} cells. The final cell density at the end of the culture, based on the 3.5-L working volume, was 4.31×10^7 cells/mL.

Based on the cell density at the end of the culture (4.31×10^7 cells/mL) and the glucose consumption

rate on the last day (22.37 g), we obtained the glucose-consumption-to-cell-density conversion ratio at the end of the culture. Assuming that this conversion ratio remained relatively unchanged over the duration of the culture, we converted the daily glucose consumption rate into daily cell density. The calculated Vero cell growth curve is shown in *Figure 2B*.

Conclusion

We have demonstrated that the Eppendorf BioBLU 5p Single-Use Vessel prepacked with Fibra-Cel disks is an excellent platform for high-density Vero cell culture. In the 3.5-L vessel, we achieved a high Vero cell density of approximately 43 million cells/mL, demonstrating great potential for Vero cell–based vaccine production. Since viral particles are much smaller than cells, they can be directly eluted from packed-bed cell culture vessels without removal of cells. In addition, as high Vero cell densities can be translated into high virus production yield, that is, high titer, the Fibra-Cel packed-bed platform is ideal for high-titer viral vaccine production. ■

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