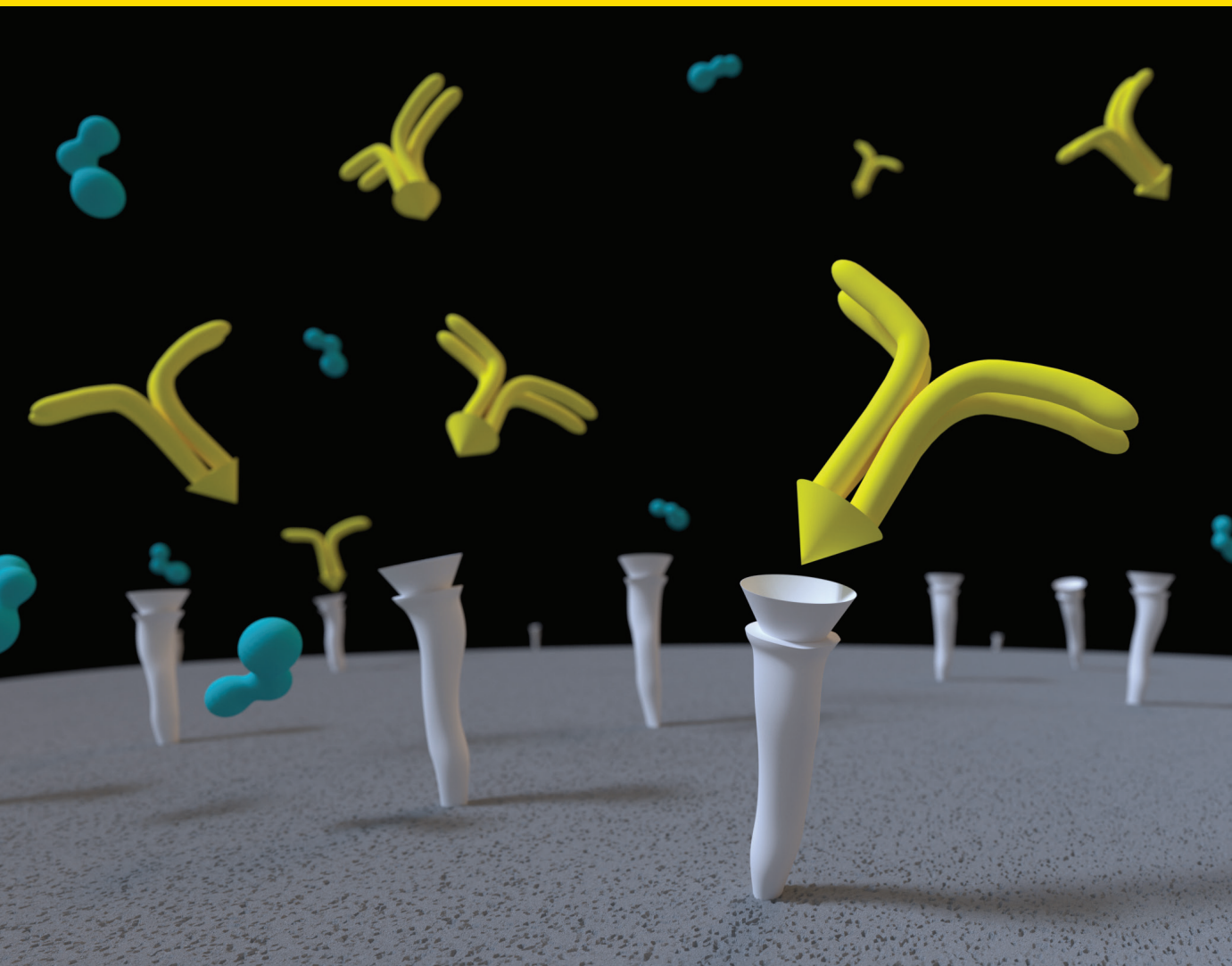


SARTORIUS

Rethinking Chromatography



BioProcess
International
SPECIAL REPORT

Process Chromatography in the Past, Present, and Future

Katy McLaughlin and Casey Mihal

Chromatography is used in the separation of almost all biomolecules, playing an essential role in ensuring that biotherapeutics are sufficiently purified and safe. It also represents a significant proportion of downstream costs in bioprocessing. As a result, it is critical that chromatography procedures be efficient and economical to reduce costs and improve patient access to life-saving therapeutics.

Here, Sartorius discusses some critical topics in process chromatography today. First, we outline key obstacles for chromatography as we move into the future of biotherapeutics. We then present a published article on the value of convective chromatography for the separation and analysis of large biomolecules. Finally, we explore strategies for increasing productivity in process-chromatography pipelines.

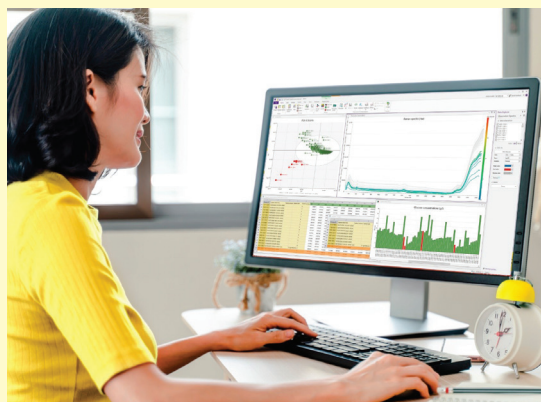
Continuing advancements in science and technology have shifted the therapeutic landscape toward new modalities and intensified production strategies. The COVID-19 pandemic certainly has accelerated this move. Supported by increased government funding (both in academia and industry) and public engagement, the rapid production of mRNA vaccines against SARS-CoV-2 has put a spotlight on the importance of accelerated development. In particular, it has highlighted how advanced modalities and supporting technologies can address new clinical indications.

NEW MODALITIES

The next generation of drugs will rely on the production of large biomolecules such as viruses (lentiviruses, adenoviruses, and adenoassociated viruses) and nucleic acids (genomic DNA, mRNA, and plasmid DNA). The distinct features of these molecules mean that typical manufacturing operations, including traditional chromatography systems and processes, are not always suitable. That introduces new obstacles and places increased pressure on biomanufacturers.

Appropriate Methodologies: In the past, biomanufacturers relied on chromatography resins to purify large molecules. Resin chromatography is suitable for proteins, but it is inefficient for nucleic

Figure 1: Comprehensive and user-friendly analytics platforms support robust downstream bioprocessing. The SIMCA-online integrated software platform monitors chromatography processes in real time using multivariate data analysis (MVDA). That provides a live, holistic view of chromatography processes that encompasses more than typical process parameters.



acids and viruses, which are too large to access resin pores fully. These large, complex molecules also are sensitive to shear stress created by tangential flow in the resin matrix. Resins have been the primary workhorse in the chromatography field for decades. Although they remain a powerful tool, resins are typically not the most effective method for solving the manufacturing challenges associated with new biotherapeutics. Alternatives must be considered.

Significant progress has been made, yet there remains a need for comprehensive solutions for the purification of large, fragile biomolecules. Now, we have new, improved chromatography matrices, technologies, and solutions to assist in the purification of complex biomolecules. However, these are not well established in the biopharmaceutical industry, and a fully platform approach — including appropriate analytics — does not yet exist. Developing a new platform to isolate a novel biotherapeutic requires significant knowledge and expertise, and the commercial production of virus- and nucleic-acid-based therapeutics is still in its infancy.

Knowledge: The speed at which the most novel therapeutics have emerged leaves much to learn

about their features, chemistry, and handling requirements. To gain access to additional expertise and technologies, biotechnology companies can employ the services of consultants or contract development and manufacturing organizations (CDMOs). The experience and resources offered by CDMOs undoubtedly has increased the number of companies that are able to undertake the production of advanced therapies and continue to drive the growth of manufacturing in this sector.

As our collective knowledge and engagement increase, more start-ups are reaching commercialization without outsourcing, opting to assemble the necessary expertise and technology in house. Both are valid paths, each with different merits. Ultimately, the decision will depend on business needs and drivers.

MAXIMIZING PRODUCTIVITY

Competition in the biomanufacturing industry puts immense pressure on drug developers to enhance their productivity by lowering costs and speeding up production. These increasing demands push manufacturers towards new production strategies that improve their efficiency, flexibility, and product quality. Although many strategies exist for maximizing process chromatography efficiency — including versatile systems, chemistries, and modalities — there are also significant barriers to their widespread implementation. Those include the speed at which the market moves, the emergence of new modalities and technologies, the absence of reliable analytic platforms, and the need for comprehensive, end-to-end purification solutions.

Dynamic Trends: The biopharmaceutical market is constantly evolving through the emergence of new modalities, technologies, and regulations. Those changes mean that drug developers need to design flexible production processes to remain competitive.

Embracing new modalities, taking advantage of the latest technologies, and ensuring that quality and safety standards are maintained all could require changes to an existing process, calling for specialized expertise and equipment. These demand their own development and optimization processes. Lack of knowledge (about a process/modality) and experience could make it tricky to maximize efficiency quickly.

The fast-moving environment of the industry means that drugs are getting to market more quickly than ever before. Regulations also can change rapidly, and failure to keep up with requirements can cause significant roadblocks. Companies have to be agile while on their journey to the clinic or commercialization to prevent bottlenecks and keep up with competitors.

Need for Robust Analytics Platforms: Careful monitoring during biopharmaceutical manufacturing helps drug developers ensure that their production processes meet the stringent quality standards that ensure patient safety. As well as testing critical quality attributes (CQAs) of a biologic once it has been recovered, real-time monitoring and in-process control of production provide valuable insights into processes and materials. Such information promotes quality, consistency, and productivity from process development to commercial manufacturing.

Although excellent analytic platforms are currently on the market (Figure 1), there is still progress to be made to support their wider application, particularly to downstream steps. One solution is the generation of truly integrated solutions that fit into a broader process. Such end-to-end platforms will include consumables (analytical columns), powerful sensors, flexible control modules, and intuitive analysis packages to support a better process understanding. These comprehensive solutions enable manufacturers to quickly identify areas in which process efficiency can be improved. Incremental process improvements are critical during continuous and intensified manufacturing.

Advanced technologies such as spectroscopic tools also are required to keep up with the increasingly strict regulations placed on biopharmaceutical manufacturers. These tools provide abundant data beyond traditional solutions, including enzyme-linked immunosorbent assay (ELISA) and high-performance liquid chromatography (HPLC), which will no longer be sufficient to demonstrate product safety. More at-line and on-line analytics will also be required to support the industry as continuous and intensified processing become increasingly adopted.

FUTURE OUTLOOK

Present issues will be solved with better technologies and an increased understanding of the molecular attributes of novel therapies. As well as exploring new technologies, manufacturers will seek to improve and intensify their existing processes to maximize the productivity of their chromatography tools.

The rest of this report outlines chromatography solutions serving the industry in the production of biotherapeutics. First, we provide evidence demonstrating the capabilities of monolithic chromatography for the production of large biomolecules. Then, we discuss the latest solutions for increasing productivity in downstream processing. 🌐

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Purification of Large Biomolecules Using Convective Chromatography

Tomas Kostelec, Rok Sekirnik, Anže Martinčič Celjar, Kristina Šprinžar Nemec, Andreja Gramc Livk, Pete Gagnon, and Aleš Štrancar, with Katy McLaughlin and Casey Mihal

Many of the challenges associated with the purification of large biomolecules point to the need for reliable analytics at every step of the journey. That is critical for complete characterization of a product, which enables drug developers to maximize productivity and ensure patient safety. Comprehensive process characterization is vital to efficient production. However, gaps in current analytical capabilities and process understanding lead to ineffective process performance, which can be costly in many cases.

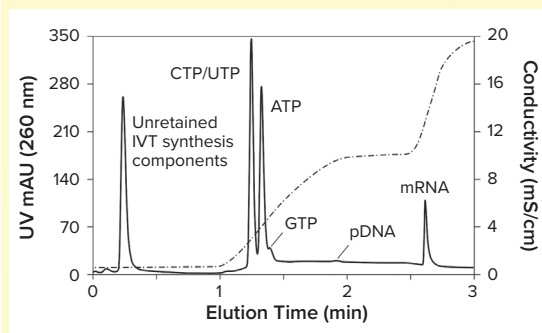
Convective analytical chromatography columns can be used to solve some of those pain points. The following article, originally published in the June 2021 issue of *BioProcess International*, presents a novel analytical method for the purification of mRNA. The results highlight the capabilities of CIMac PrimaS analytical columns to separate in vitro transcription (IVT) components and deliver critical information about mRNA synthesis kinetics.

At-line and on-line analytics require rapid and accurate assays that generate insights into a process at all stages. Current analytical methods for large biomolecules typically require significant preparation work and are too lengthy for use within a process. New analytical solutions that offer rapid results while still providing accurate in-process information are vital for solid process characterization. Convective chromatography presents an opportunity for users to develop fast and reliable assays that can be used at line for real-time monitoring of their process at multiple stages.

A COMPLEX PROCESS

COVID-19 has focused a spotlight on the ability of mRNA technology to accelerate vaccine development and approval (2). That same technology can hasten development and approval of other therapeutic classes, including cancer immunotherapy, protein replacement, and gene therapy. Fulfilling those opportunities imposes significant challenges on

Figure 1: In vitro transcription (IVT) analysis using CIMac PrimaS columns after four hours of incubation (see “IVT Characterization” box for conditions)



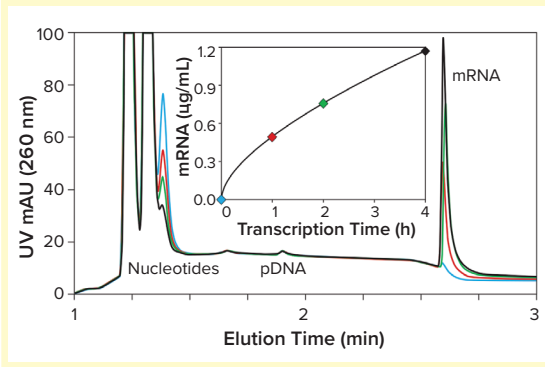
process developers and manufacturers to improve existing processes. Scale-up to produce millions of doses (tens of kilograms) compounds those challenges. Furthermore, every step of the journey requires high-performance analytical methods to ensure patient safety and maximize productivity.

This complex process begins with a DNA plasmid (pDNA) produced in *Escherichia coli* and its subsequent purification. The process continues with in vitro transcription (IVT), followed by purification of the mRNA.

With the target DNA sequence defined and inserted into a plasmid, amplification is performed in *E. coli* to produce a pDNA template. Lysis is followed by precipitation with calcium chloride to reduce contamination by host-cell RNA. Plasmid purification by anion-exchange chromatography (AEC) and hydrophobic-interaction chromatography (HIC) follows to remove endotoxins, host-cell proteins, host-cell RNA, pDNA fragments, and aggregates (3). Then, supercoiled plasmid is linearized with an enzyme that requires subsequent removal.

Approaches to **transcription** of linearized plasmids are still evolving, but they essentially involve combining plasmids with the raw materials (nucleotides and enzymes) for synthesizing RNA (4).

Figure 2: Synthesis kinetics of mRNA as measured by anion-exchange HPLC analysis (CIMac PrimaS columns, see “IVT Characterization” box to the right)



IVT CHARACTERIZATION WITH CIMAC PRIMAS COLUMNS

Column: CIMac PrimaS (100 μ L, Sartorius Stedim Biotech, Ajdovščina, Slovenia; cat. no. 110.5118-2)

Flow Rate: 2 mL/min

Buffers: Buffer A was 50 mM HEPES, pH 7.0; buffer B was 50 mM HEPES, 200 mM sodium pyrophosphate, pH 8.5.

The column was equilibrated with buffer A. Sample was injected, then washed with buffer A. Elution was performed with a linear gradient to 20% B, then another linear gradient segment to 50% B. The column was cleaned with 100 mM NaOH and 2.0 M NaCl, then regenerated with 1.5 M HEPES (pH 7.0).

High efficiency of transcription is important because the more copies of mRNA that can be obtained from each pDNA molecule, the lower will be the ratio of product to contaminants in a completed IVT mixture. IVT production of mRNA for therapeutic use is complicated further by the need to stabilize the 5' ends by adding a capping reagent either cotranscriptionally or posttranscriptionally.

Purification needs to remove the raw materials used for synthesis along with mRNA impurities such as aborted (short) transcripts, double-stranded RNA, and aggregates. Technologies for doing so also are evolving. As protein purification did in the past, the field of mRNA is moving away from precipitation methods toward chromatographic methods that achieve higher recoveries and purities, more predictable scalability, and better reproducibility. Multiple options are available for both capture and polishing, including affinity, anion-exchange, hydrogen-bonding, hydrophobic-interaction, and reversed-phase chromatography methods (5-7).

THE ANALYTICAL BOTTLENECK

The complexity of the entire processing chain from *E. coli* cell paste to purified mRNA is compounded by the less visible but equally important task of characterizing each step. It is fundamental to maximizing productivity and documenting the purity necessary to ensure patient safety. Characterization of IVT, especially as it applies to optimization, is particularly challenging because it must include interactions among many variables. The process begins with assessing the quality of reagents. Plasmids can carry proteins, endotoxins, and other residual contaminants. Similarly, enzymes can include stabilizing additives unsuitable for in vivo therapeutics, sometimes degraded to varying degrees (5-7). Nucleotides need to be provided in

adequate quantities so that they do not limit transcription. The same is true of capping reagents. The ratio of enzymes to plasmids must be balanced carefully. Monitoring buffer conditions, time, and temperature also is essential. Some reagents are extremely expensive, so they need to be used under the most favorable conditions.

In the IVT process, the conditions outlined above require optimization or confirmation for every new mRNA construct. Common analytical methods used in IVT development provide information about one parameter at a time (e.g., agarose gel electrophoresis to confirm the presence of RNA species or sodium dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to detect protein contaminants). Some of those methods are also time-consuming and lack automation, which extends development timelines. New analytical tools can potentially disrupt the way mRNA processes are developed and monitored.

ELIMINATING THE ANALYTICAL BOTTLENECK

High-performance liquid chromatography (HPLC) has a long history of providing analytical data to support development and manufacture of biologics, and it has unique capabilities for mRNA development. Benefits of HPLC include rapid results, high resolution, and quantitative monitoring, with minimal sample volume requirements. Upstream processes such as IVT reactions, where the target molecule is produced and reagents are consumed, would benefit strongly from implementation of HPLC. AEC has been a foundation HPLC method for proteins and nucleic acids, but large mRNA molecules can be problematic because they do not elute from traditional AEC media at ambient temperature. New anion exchangers have begun to overcome that limitation.

Figure 1 illustrates ambient temperature fractionation of an mRNA IVT mixture at four hours from the start. The profile was produced with an ascending salt–pH gradient (for conditions, see the box at right). mRNA is well separated from other IVT components, which elute at earlier retention time. The profile also provides a snapshot of the levels of nucleotides and their proportions compared with those of pDNA and mRNA. The 100- μ L column format minimizes sample consumption, typically requiring sample volumes of only 25 μ L (10–30 ng RNA). At the working flow rate of 20 column volumes (CV) per minute, a complete analytical cycle requires five to eight minutes. Profiles of this type provide the quantitative foundation necessary for evaluating complex reaction kinetics. Figure 2 overlays IVT profiles from samples taken at 30 seconds, one hour, two hours, and four hours. Consumption of nucleotides is revealed in parallel with increasing concentration of mRNA. Analytical series of this type can be used to characterize quickly and efficiently the influence of each process variable desired.

PrimaS elution conditions also can be configured to highlight other contaminant subsets. As shown in Figures 1 and 2, plasmid DNA (pDNA) binds much more weakly than single-stranded mRNA (ssRNA) does. Double-stranded RNA (dsRNA) elutes later than pDNA but before mRNA (3–5). Weak binding also enables elimination of double-stranded species in a salt step, whereas elution of mRNA requires an increase of pH. PrimaS columns have the further ability to separate mRNA according to size in low-conductivity pH gradients, enabling discrimination of short transcripts and aggregates.

The role of AEC also extends to pDNA. Other anion exchangers are broadly used for initial monitoring of pDNA. Elution of pDNA from DEAE, for example, takes place at ambient temperature. The supercoiled plasmid is clearly differentiated from open-circular and linear conformations and also from host RNA, aggregates, and proteins (7). AEC also provides an additional benefit for both pDNA and mRNA. Implementation of this new analytical method can provide improved process understanding, leading to faster, more robust and consistent manufacturing.

REFERENCES

1 Kostelec T, et al. Eliminating the Analytical Bottleneck in Production and Purification of mRNA. *BioProcess Int.* 19(6) 2021: 70–71; <https://bioprocessintl.com/sponsored-content/production-and-purification-of-mrna>.

2 Wadhwa A, et al. Opportunities and Challenges in the Delivery of mRNA Based Vaccines. *Pharmaceutics* 12,

PrimaS columns have the further ability to separate mRNA according to size in low-conductivity pH gradients, enabling **DISCRIMINATION** of short transcripts and aggregates.

2020: 102; <https://doi.org/10.3390/pharmaceutics12020102>.

3 Černigoj U, Štrancar A. Scale-Up of Plasmid DNA Downstream Process Based on Chromatographic Monoliths. *DNA Vaccines: Methods and Protocols*. Sousa A, Ed. Humana: New York, NY, 2021; 167–192.

4 Hadas Y, et al. Optimizing Modified mRNA In Vitro Synthesis Protocol for Heart Gene Therapy. *Mol. Ther. Met. Clin. Dev.* 14, 2019: 300–305; <https://doi.org/10.1016/j.omtm.2019.07.006>.

5 Gagnon P, et al. Two New Capture Options for Purification of Large Messenger RNA. *Cell and Gene Therapy Insights* 6(7) 2020: 1035–1046; <https://www.biaseparations.com/en/library/publications/1090/two-new-capture-options-for-improved-purification-of-large-mrna>.

6 Gagnon P. A New Runway for Purification of Messenger RNA. *BioProcess Int.* 18(10) 2020: 36–45; <https://bioprocessintl.com/wp-content/uploads/2020/10/18-10-Gagnon.pdf>.

7 Gagnon P. *Purification of Nucleic Acids: A Handbook for Purification of Plasmid DNA and mRNA*. BIA Separations: Ajdovščina, Slovenia, 2020; ISBN 978-962-95042-0-8; <https://www.biaseparations.com/en/products/monolithic-columns/books/226/purification-of-nucleic-acids-a-handbook-for-purification-of-plasmid-dna-and-mrna-for-gene-therapy-and-vaccines>. 🌐

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Maximizing Downstream Productivity

What's Next for Your Process?

Katy McLaughlin and Casey Mihal

Biomanufacturers must keep up with intense pressure in the constantly evolving biotherapy field, requiring them to seek out solutions for increased productivity and flexibility. Efficient manufacturing pipelines allow drug developers to maximize their output, keep costs low, and remain competitive.

Getting the most out of each step is essential to building an efficient process. Much of the interest in applying intensified production strategies relate to upstream processing. However, manufacturers are increasingly exploring alternative chromatography methods to enhance efficiency and minimize costs in their downstream steps. One example of such a modification is multicolumn chromatography (MCC), which supports continuous bioprocessing.

CONTINUOUS CHROMATOGRAPHY

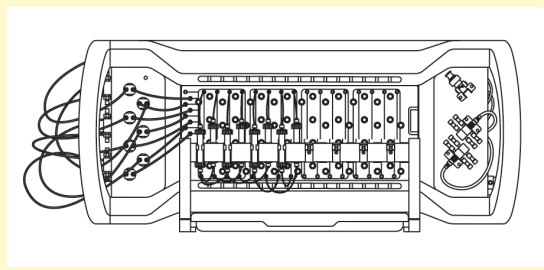
Traditional batch chromatography can limit productivity. The single-column approach creates production bottlenecks, requires excess resin to prevent breakthrough, and has a limited binding capacity. Solutions that lower the volume of necessary resins while maximizing recovery and speed are desirable for limiting costs and maximizing efficiency because the cost of resins represents a significant proportion of downstream process expenses.

MCC, also called *continuous chromatography*, uses a series of smaller columns assembled to allow for serial loading, enabling complete loading of each column (Figure 2). This arrangement maximizes binding, increases resin utilization, and enhances a system's overall capacity. Additionally, columns can be processed individually, allowing several steps (loading, washing, elution, regeneration, equilibration) to be performed simultaneously and thus speeding up the downstream process.

ADOPTION CHALLENGES

Overall, MCC can increase productivity by up to five-fold, reduce resin requirements by almost 80%, and help limit downstream processing bottlenecks (1). Consequentially, continuous chromatography supports a more sustainable, efficient process, allowing drug

Figure 1: Compared with traditional batch processing, the series of smaller columns present in a multicolumn chromatography (MCC) system can enhance productivity and reduce costs.



developers to keep up with the changing demands of the biopharmaceutical industry.

Although the potential for MCC to contribute to a more efficient process is clear, it is not yet widely adopted. Continuous bioprocessing requires a high degree of process understanding supported by platforms enabling monitoring, control, and analysis of each step. Fully integrated, automated, and single-use (SU) technologies are also essential to achieving the productivity improvements offered by continuous chromatography. Combined with the speed at which the biopharmaceutical market moves, such factors are likely barriers to MCC adoption in many facilities.

CONTINUOUS CHROMATOGRAPHY SOLUTIONS

As the biopharmaceutical landscape continues to develop, technologies supporting next-generation production processes and creating advanced modalities are also emerging. These include improved sensors and process analytical technology (PAT) for monitoring and control and the application of SU technologies to support increased consistency.

PAT AND DATA ANALYTICS

A robust manufacturing process relies on careful monitoring and control of critical process parameters (CPPs) and quality attributes (CQAs). That requires reliable sensors and analyzers to measure quality attributes throughout the continuous-chromatography process. An ideal analytics platform will also be

END-TO-END platforms are likely to become significant parts of efficient chromatography processes in **NEXT-GENERATION** facilities that perform continuous, intensified bioprocessing.

supported by effective data acquisition, management, and storage capabilities as well as integrated data analysis software.

The insights provided by analytical technologies enable users to enhance reproducibility and successfully automate their chromatography pipelines. Integrated, plug-and-play technologies with intuitive interfaces are desirable because they can be seamlessly incorporated into an existing setup and can immediately unlock valuable information.

Comprehensive PATs for chromatography will include real-time monitoring of multiple parameters such as purity, kinetics, and molecule conformations. Advancing technologies will help deliver novel data and improved process understanding. One fast-emerging example is the more widespread use of Raman spectroscopy. Supported by quality by design (QbD) principles, Raman spectroscopy is well suited to capturing high-resolution information during continuous chromatography. It provides rapid information on CQAs, which can change quickly during downstream processing.

PATs and high-performance analysis tools help drug developers to maximize their product quality and process robustness. By supporting close monitoring and incremental process improvements, such tools ultimately lead to a more intensified and efficient process.

SINGLE-USE TECHNOLOGIES

SU technologies increasingly are employed in biopharmaceutical manufacturing. Delivering consistency, speed, and reliability, SU solutions support flexible, efficient production processes. They are a critical part of many next-generation facilities.

In chromatography, SU technologies include pre-packed columns, cassettes, and chromatography kits. Although single-use tools are used widely in some manufacturing areas, end-to-end SU solutions have yet to reach their full potential in the industry.

Fully closed comprehensive platforms will include the chromatographic device and sterile connectors to minimize operator involvement and support full

automation. Such solutions will also cut costs and decrease time associated with cleaning, allowing users to remain flexible and agile in their manufacturing strategies. End-to-end platforms are likely to become a critical part of an efficient chromatography process in a next-generation facility employing continuous, intensified bioprocessing.

LESSONS LEARNED FROM PROTEINS

Earlier biotherapeutic modalities, such as recombinant proteins and monoclonal antibodies (MAbs), have relied heavily on traditional chromatography for their efficient purification. Improvements in downstream processing have made these processes more efficient and reliable, helping drug developers to remain competitive and respond to ever-changing trends in the global market. Such trends include increasing competition and the scientific advancements that lead to new modalities such as therapies based on nucleic acids.


Improvements include new or resurgent technologies to support the isolation of large biomolecules and the trend toward continuous processing to maximize process productivity. These enhancements are facilitated by technologies supporting automation, analytics, and SU manufacturing.

Future challenges will include combining process improvement strategies with large biomolecule production scenarios to bring intensified production to the next generation of biologics. The lessons learned from the establishment of protein-based therapies will serve the industry well in the dynamic biotherapeutics market. The release of new modalities and the necessity for more efficient production strategies will be supported by advanced technologies for purification and analysis.

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REFERENCE

1 Mao X. *BioSMB Process System: Scale-Up of Multi-Column Chromatography*. Sartorius: Göttingen, Germany, 18 January 2021; <https://www.sartorius.com/download/699288/biosmb-process-system-application-note-en-b-2475299-sartorius-1--data.pdf>. 

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