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Biopharmaceutical Manufacturing: Historical Perspectives and Future Directions

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Abstract

This review describes key milestones related to the production of biopharmaceuticals—therapies manufactured using recombinant DNA technology. The market for biopharmaceuticals has grown significantly since the first biopharmaceutical approval in 1982, and the scientific maturity of the technologies used in their manufacturing processes has grown concomitantly. Early processes relied on established unit operations, with research focused on process scale-up and improved culture productivity. In the early 2000s, changes in regulatory frameworks and the introduction of Quality by Design emphasized the importance of developing manufacturing processes to deliver a desired product quality profile. As a result, companies adopted platform processes and focused on understanding the dynamic interplay between product quality and processing conditions. The consistent and reproducible manufacturing processes of today's biopharmaceutical industry have set high standards for product efficacy, quality, and safety, and as the industry continues to evolve in the coming decade, intensified processing capabilities for an expanded range of therapeutic modalities will likely become routine.

INTRODUCTION

Recombinant DNA technology: methods to artificially assemble pieces of DNA from various organisms for delivery and expression in cells

Recombinant proteins: proteins produced using recombinant DNA technology

Cellular and gene therapies (C&GTs): therapies that modify or manipulate gene expression or cellular properties for the treatment of disease

Monoclonal antibodies (mAbs): homogeneous antibodies originating from a single B-cell targeting a single epitope

Primary cells: cells obtained directly from animal or human tissue with a limited life span in vitro

Cell line: cells originating from a single ancestor that can be grown in vitro for an extended period of time

Biopharmaceuticals represent a diverse class of pharmaceutical products that has grown to encompass any biological product that is produced by or from living organisms using recombinant DNA (rDNA) technology, including recombinant protein therapeutics, vaccines, and cellular and gene therapies (C&GTs) (1). Over the past four decades, more than 300 biopharmaceutical products have reached the market, with more than 100 product approvals between 2015 and July 2018 (2). The biological origin of these products can offer novel mechanisms of action for treating a variety of indications (such as cancers, autoimmune diseases, genetic disorders, or infectious diseases) as compared to small-molecule drugs, and the therapeutic recombinant proteins available now range from hormones and growth factors to interferons and enzymes. Monoclonal antibodies (mAbs) in particular have seen rapid growth as treatments, as their ability to bind targets with high affinity and specificity and then activate the immune system are tasks that can be difficult to achieve with small molecules. In 2021 alone, 115 mAbs entered the clinic, primarily for oncological indications (3). Of the 20 top-selling therapeutics in 2019, 9 were mAbs, collectively bringing in $75 billion in earnings, and several other potential blockbuster mAbs were approved in 2020 (4, 5). In April 2021, the US Food and Drug Administration (FDA) approved its one-hundredth mAb, 35 years after the approval of the first, and the mAb market is predicted to reach $300 billion by 2025 (4, 6). Within the last five years, the regulatory approvals of C&GTs have created personalized treatment options, and potentially long-term cures, for previously difficult-to-treat diseases (2).

Whereas therapeutics produced using rDNA technology started reaching patients only in the 1980s, biological products, such as porcine-derived insulin and poliovirus vaccines, were well-established. These early products were derived mainly from animal tissues, serum, or primary cell lines, and the variability inherent to these production platforms led to low product purity and efficacy compared to today's standards. Leveraging knowledge from existing manufacturing processes played a major role in the biopharmaceutical (biopharma) industry's rapid growth, and unit operations used today were influenced by the technologies established in the first half of the twentieth century, many of which operate based on fundamental principles familiar to chemical engineers. Incremental improvements upon these foundational technologies have led to modern biopharma processes that have set new standards of consistency and reproducibility, leading to improved product efficacy, quality, and safety.

The general life cycle of a biopharmaceutical product begins with discovery, during which thousands of initial candidate molecules are generated and screened for activity against a chosen biological target. Top-performing candidates undergo optimization to improve efficacy and safety testing both in vitro and in vivo. Products chosen for further study in humans progress to process development (PD) groups that are responsible for developing the production processes required to generate material at sufficient scale to supply clinical trials and eventually meet commercial demand. This review presents a historical perspective on PD and manufacturing of recombinant protein therapeutics and highlights the key advancements that have allowed the industry to reach its current state, specifically for cell line development, upstream cell culture processes, and downstream purification processes, ending with drug substance (**Figure 1**). Drug product manufacturing and product stability, although crucially important to the industry, are not discussed here and have been reviewed elsewhere (7). While the recombinant protein portfolio covers a variety of protein classes, the large economic demand for mAbs has driven much of the manufacturing and process improvements made across the biopharma industry, with findings from mAb research applied to other products. Because of their profound impact on the growth and direction of the biopharma industry, this review focuses primarily on work done in the context of mAb production.

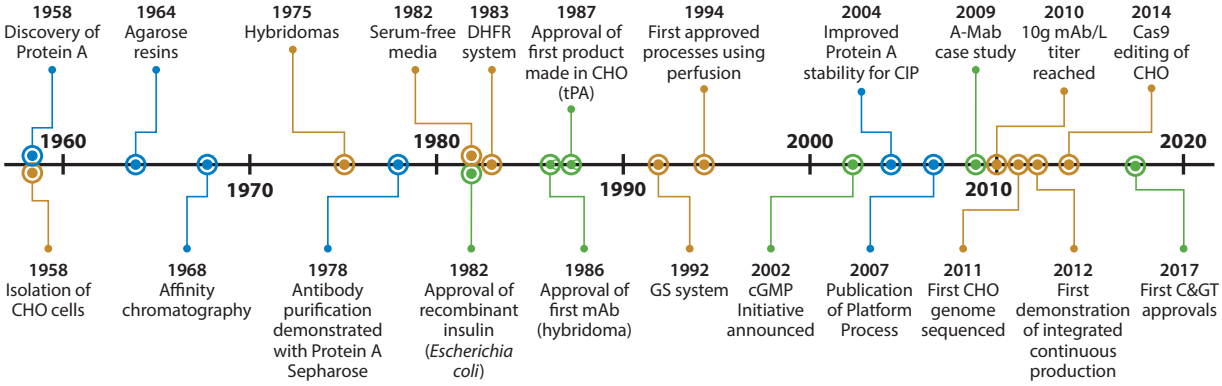

Figure 1

Notable milestones and revolutionary discoveries in biopharmaceutical manufacturing, including upstream technology advances (*yellow*), downstream technology advances (*blue*), and industry milestones (*green*). Dates are based on publication dates. Abbreviations: cGMP, current good manufacturing practice; CHO, Chinese hamster ovary; CIP, clean-in-place; DHFR, dihydrofolate reductase; GS, glutamine synthetase; mAb, monoclonal antibody; tPA, tissue plasminogen activator.

EMERGENCE OF THE INDUSTRY: ENABLING TECHNOLOGIES AND EARLY PROCESSES (~1960s–1989)

Product Discovery and Optimization: Harnessing Recombinant DNA Technology

The emergence of rDNA technology, which allowed scientists to assemble DNA from different organisms to generate and deliver new genetic constructs into a cell host of choice, created new and unprecedented methods for discovering, engineering, and producing biopharmaceuticals. To obtain a product of interest, scientists no longer had to rely on primary cells derived from animals, many of which were difficult to isolate, were challenging to grow at large scale, produced low quantities of product, and were inherently variable. rDNA technology instead offered scientists the opportunity to produce humanlike proteins in cell types that were easier to grow at large scale and produced enough product to meet the increasing demand for medicines. Genentech's successful production of human insulin in *Escherichia coli* using rDNA technology in 1979 marked the first major application of rDNA by demonstrating that human proteins with therapeutic potential, even complex proteins such as heterodimeric insulin, could be produced in non-native hosts and successfully purified to generate a functional medicinal product with a favorable efficacy and safety profile (8, 9). The positive clinical trial data supported the FDA's approval of the first recombinant human insulin product in October 1982 (9), a milestone decision that signaled the acceptance of recombinantly produced biotherapeutics and ushered in the new era of the biopharma industry. New recombinant therapies for other protein classes using *E. coli* production platforms were approved in quick succession—Genentech followed their insulin success by winning approval for recombinant human growth hormone in 1985, and two recombinant interferons were approved in 1986 (2).

rDNA techniques also played a crucial role in the commercialization of antibody therapeutics, as they enabled the simultaneous discovery and production of mAbs through hybridoma technology, first published in 1975 (10). Antibody therapies had already been used for decades, yet they relied on polyclonal antibodies derived from pooled serum samples from many donors (human or animal), which created a variable and heterogeneous mixture of antibody structures derived from many B-cell populations that was difficult to characterize (11). Köhler & Milstein's (10) hybridoma

Polyclonal antibody: mixture of heterogeneous antibodies produced from different B-cells that target different epitopes on the same antigen

method presented a way to make monoclonal antibodies instead—chemically identical antibodies originating from the same B cell. The reproducibility of the hybridoma technique was key for the widespread implementation of antibody therapies: Once a hybridoma cell line was established, one antibody with a defined structure and known efficacy could be produced reliably through cell culture. Early hybridomas found use in generating mAbs for clinical diagnostics and for basic immunological research, but when three mAbs that targeted T-cell surface antigens were discovered in 1979, researchers realized that these antibodies could also serve a therapeutic purpose (4, 12). Clinical testing of one of the mAbs showed that it was an effective immunosuppressant to combat acute organ rejection following transplantation (13), and Orthoclone OKT3 muromomab-CD3, as the product was eventually named, was the first commercialized mAb product and the first product developed using hybridoma technology (2).

Early hybridoma cultures were derived from murine cell lines and produced murine-like antibodies, which differed in structure from human antibodies enough to cause two major problems for early mAb products: First, murine antibodies were not easily recognized by the human immune system, and second, they could elicit immune responses in patients, resulting in the generation of human anti-mouse antibodies (HAMAs) (14). Reports of what became known as the HAMA response were published as early as 1983 and highlighted the need for improvements in antibody frameworks to reduce immunogenicity in patients and improve effector functions (15, 16). Using rDNA techniques, methods for chimerization, the fusion of murine-derived variable regions to human immunoglobulin constant regions, and humanization, the grafting of murine-derived complementarity-determining regions onto human variable regions, emerged to address this problem while simultaneously establishing common frameworks for mAb products (17–19). The establishment of a polymerase chain reaction technique to obtain antibody genes directly from primary or hybridoma cells in 1989 expanded upon available techniques for isolation and engineering of antibody genes (20). This new technique was especially profound in that the antibody sequences obtained could be expressed and screened for affinity using alternative cell platforms, then engineered for reduced immunogenicity or improved function as needed. These discoveries eventually led to methods such as phage display (21, 22) and recombinantly generated in vitro libraries that have further evolved into the platforms used for antibody discovery today (23). Obtaining an antibody sequence or expression vector directly from the discovery platform also enabled easier generation of antibody-producing cell lines in a host more amenable to large-scale production.

Cell Line Development: The Emergence of CHO Cells

Many early biopharma products relied on the *E. coli* K12 strain that had been heavily engineered to minimize or eliminate any risk to patients (9), yet bacterial systems were unable to reliably produce soluble mammalian proteins in large quantities. To maximize yields, microbial processes required the extraction, re-solubilization, and refolding of the protein of interest from inclusion bodies, increasing process complexity (24). The lack of required enzymatic pathways and compartmentalization for mammalian-like glycosylation also limited the types of proteins that could be produced using microbial hosts (25). As a result, the emergence of mammalian cell lines that could safely and effectively produce large quantities of protein with humanlike posttranslational modifications was critical for the growth of the biopharma industry, with Genentech's tissue plasminogen activator (tPA), produced in suspension Chinese hamster ovary (CHO) cells, and Amgen's erythropoietin (EPO), produced in adherent CHO cells, as milestones that established safety and efficacy of CHO-produced therapeutic proteins (2).

In 1958, Puck et al. (26) first isolated and cultured CHO cells to be used as a model cell line for genetic studies. Puck et al. (26, p. 950) observed that the adherent cells obtained from Chinese hamster (*Cricetulus griseus*) ovary tissue were "particularly hardy and reliable," maintaining continuous cultures for more than 10 months with no observable differences in morphology. CHO cells had a relatively low chromosome number ($2n = 22$), and Puck's team observed no change in karyotype during their initial 10 months of cultivation. The strong performance of the Chinese hamster–derived cells drove further characterization and cloning, including the derivation of the familiar CHO-K1 cell line in 1968 (27). Importantly, CHO cells were adapted to suspension cell culture (CHO-S, generated in 1971), allowing them to grow at large scales in stirred tank bioreactors, and modified to generate cell lines with useful metabolic deficiencies for use with auxotrophic selection systems, such as the dihydrofolate reductase (DHFR)/methotrexate system. The creation of two CHO host cell lines deficient in DHFR activity (CHO-DXB11, generated in 1980, and CHO-DG44, generated in 1983) that supported high expression of transgenes, coupled with CHO's robust growth profile, made the cells an attractive host for the growing biopharma industry (28–30).

Transgene: a gene that has been transferred from one organism to another

Gene amplification: a process to increase the copy number of specific genes in a cell's genome

Upstream Process Development: Leveraging Knowledge from Established Processes

Much of the foundational work for large-scale mammalian cell culture processes using both adherent and suspension cell lines was done for the nonrecombinant production of viral vaccines for livestock, human interferons, or antibiotics, and principles from these industries were applied to the cell culture bioreactors used by the early biopharma industry (31–33).

To provide adherent cells with the necessary growth surface, early processes used roller bottles, which were partially filled with media and slowly rotated, allowing cells to adhere (34). Scale up of these processes was straightforward by simply increasing the number of bottles used, yet increased throughput also required increased plant space and the adaptation of existing protocols to accommodate more bottle manipulations (35). Microcarrier culture technology, where cells grow on the surface of suspended beads, provided an alternative for the growth of adherent cell lines and was first used industrially in 1975 (36, 37). Microcarrier cultures combined the convenience of stirred tanks with the required adherent surface for the cells; however, first-generation microcarriers exposed the cells to high levels of agitation, foaming, and shear forces (35).

The transition from adherent mammalian cell culture to suspension cell culture in the biopharma industry was driven by growing market demand for increased quantities of recombinant protein therapeutics. For example, the high dose required for tissue plasminogen activator led Genentech to adapt an adherent producer CHO cell line to suspension culture, eventually reaching the 10,000-L scale and a titer of 50 mg/L (27, 38); however, adaptation of CHO cells to suspension culture was challenging initially given the limited availability of specialized media formulations. One benefit of mAb-producing hybridomas was that they could be grown in suspension culture without adaptation, making the optimization of these processes critical for early mAb producers to reach the quantities required by predicted market demand. Significant work was done to understand and model the growth and metabolite kinetics of suspension hybridoma cultures, and studies comparing the effects of gene amplification strategy, feed composition, metabolic demand, and bioreactor mode on titer resulted in steady increases in culture productivity (39–41). Owing to concerns regarding the shear sensitivity of cells, some hybridoma processes opted to use airlift bioreactors, a design in which gas is bubbled through a draught tube as a mechanism to circulate the culture liquid, at scales up to 1,000 L (33). Manufacturers eventually realized that mammalian

cells were more tolerant to high shear than was suspected originally, and mammalian cell culture adopted many techniques similar to microbial fermentation. Fed-batch processes in stirred tank bioreactors emerged as the preferred mode for production of mAbs using hybridomas, reaching titers of >1 g/L by the early 1990s (41). PD groups then shifted focus to better understanding the scale-up and control parameters required to increase mammalian cell culture processes beyond the 10,000-L scale, again using processes for nonrecombinant biological products as precedents (33).

Fed-batch: a cell culture process where cells are routinely provided with additional nutrients to support growth

The development of improved media formulations was crucial for achieving the high-density suspension cell cultures necessary for recombinant protein production. Many mammalian cell types required supplementation with animal serum, typically fetal bovine serum, but animal-derived products carried inherent risks, including undefined variability and transmission of adventitious agents (viruses or transmissible spongiform encephalopathies) (34, 42). The first serum-free media recipe (Ham's nutrient mixture F12) was published in 1965 for the expansion of clonal CHO cells but was not suited for reaching cell densities greater than 1×10^5 cells/mL (43, 44). Murakami's 1982 identification of four essential cell culture additives for the replacement of serum (insulin, transferrin, ethanolamine, and selenium, together known as ITES) improved the performance of serum-free media, and continued refinement of nutrient levels in conjunction with the ITES additives led to the widely used enriched RDF media formulation, published in 1985. The enriched RDF basal formulation formed the basis for many early mammalian cell culture media and has been progressively optimized by the biopharmaceutical community (44–46).

Downstream Process Development: Improving Chromatography Resins

Partition chromatography was first described in 1941, but its application to large molecules like proteins was initially limited owing to the lack of appropriate resin matrices prior to the 1950s (47). The emergence of porous, hydrophilic matrices, such as cellulose- and dextran-based ion exchange (IEX) media, in the late 1950s and early 1960s demonstrated the potential of chromatography-based separations for protein purification (48–50). These matrices found early use in the purification of serum-derived immunoglobulin products, which relied heavily on IEX steps and fractionation methods (51). Dextran-based resins, however, were not ideal for large-scale processing, because they deformed easily and required low flow rates to avoid high back pressure. The discovery of agarose as a resin matrix in 1964 addressed this problem, as agarose beads were shown to have superior flow properties compared to dextran matrices (52). These findings gave rise to new resin products, such as Pharmacia Fine Chemicals' Sepharose matrix in 1967, cross-linked agarose in 1975, and eventually Sepharose Fast Flow in 1985, that could accommodate the higher flow rates needed for process-scale chromatography (51). Despite advances in resin chemistry, the purification processes for early biopharma products still relied on additional unit operations and a variety of chromatographic modes, such as size-exclusion chromatography, reverse-phase high-performance liquid chromatography, and salt precipitation (51, 53).

The demonstration that a specific, biological interaction between two molecules could be used as a chromatographic purification method was a crucial discovery for the emerging biopharma industry. The first documented affinity chromatography process purified an enzyme using a resin with the enzyme's competitive inhibitor covalently attached to the resin surface, demonstrating the potential for highly selective purification (54). Work on characterizing Protein A, a protein isolated from *Staphylococcus aureus* with a known affinity for immunoglobulins, was already underway, and Protein A was first proposed as a ligand for the purification of antibody products in the early 1970s (55–58). Pharmacia Fine Chemicals released the first commercialized Protein A resin, Protein A Sepharose, in 1975, and its ability to purify immunoglobulins from mouse serum

with nearly 100% yield was demonstrated in 1978 (51, 59). Early Protein A resins were expensive, and processing conditions were limited by the resin matrix, but progressive improvements in the matrix rigidity, bead size, and loading capacity of Protein A resins promoted their adoption into commercial processes, including the production of OKT3 (60).

GROWTH AND EXPANSION: OPTIMIZATION AND ESTABLISHMENT OF PLATFORM PROCESSES (~1990–2009)

The success of the biopharma industry's early products and the demonstration of the capabilities of rDNA technology sparked a rapid increase in the rate of new product trials, submissions, and approvals throughout the 1990s and 2000s (2). Progress made in manufacturing technology during this time period was motivated by two main drivers: (*a*) the need for increased process output to accommodate demand and dosage requirements (especially for mAb therapies), which was addressed through increased process productivity and scale, and (*b*) changes to regulatory oversight of the biopharma industry that emphasized the need for risk mitigation strategies and improved process understanding.

Cell Line Development: Finding Stable, High-Producing Cell Lines

Advances in cell line development focused on optimizing generation and screening workflows for isolating high-producing cell lines. Integration of the transgene into a host cell using an auxotrophic selection system remained the preferred method for generating stable cell lines, especially in CHO, and the publication of the glutamine synthetase (GS)/methionine sulfoximine selection system in the early 1990s provided an alternative metabolic selection and gene amplification system to the DHFR platform that did not require use of genotoxic methotrexate (61, 62). Typical workflows for isolating high-producing clones using these systems involved delivering the transgene of interest along with an additional copy of the DHFR or GS genes, then selecting cells with increasing amounts of a selection agent to enrich for cells that integrated, and subsequently amplified, the transgene cassette randomly throughout their genomes. Single cell clones could then be isolated from the selected cell populations and screened for the highest expressors. Early mAb-producing cell lines generated using these methods were shown to reach cellular productivities of 50 pg/cell/day, resulting from up to 10 copies of the transgenes; however, expression levels could vary widely between clones due to the randomness of the integration location(s), requiring significant effort to identify high-producing cell lines (63, 64). In addition, cell lines also had to be capable of maintaining expression over time, as cell lines generated through random integration were found to be susceptible to production instability across protein classes (65). Early reports showed tPA-expressing CHO cell lines exhibiting declines in productivity after extended passage in the absence of a selective agent (30) and murine hybridomas losing productivity during adaptation to serum-free media (66). These declines in productivity were attributed to either transgene copy number loss or decreased transcriptional efficiency, with studies finding that the mechanisms driving instability varied across cell lines producing a variety of products (65) and that even cell populations derived from a single parent clone were heterogeneous for transgene copy numbers, messenger RNA (mRNA) levels, productivity, and stability (63, 67). Copy number loss and gene silencing were attributed to positional effects, whereby the local genomic environment around the integrated transgene can impact expression, and genomic instability during gene amplification, leading to chromosomal rearrangements and a dramatically altered genomic landscape (68–70). Early solutions to improve production stability at the transcriptional level involved modifying transgene cassettes with regulatory DNA elements (such as scaffold/matrix attachment regions, insulators, and ubiquitous chromatin opening elements) designed to negate silencing effects or

Stable cell line: a cell line in which a transgene has been inserted into the cell's genome

Clone: an isolated single cell which is expanded to generate a cell line

Cellular productivity: the amount of product that the average cell makes over time, typically reported in pg/cell/day

Random integration: a method for inserting a transgene at a randomly chosen location in the cell's genome

Production instability: a phenomenon in which a cell line loses the ability to express a transgene over time

improve the structural stability of surrounding chromatin (34). By the early 2000s, it was recognized that integrating transgenes at a transcriptionally active genomic location could alleviate production instability, and early efforts for site-specific integration (SSI) focused on using native homology-directed repair (HDR) pathways to insert transgenes at a chosen location (71). However, HDR was found to be inefficient (72), and the discovery of various site-specific recombinase systems capable of more efficient and targeted integration of transgenes through recombinase-mediated cassette exchange (RMCE) shifted focus to these technologies (73).

Although maintaining transcriptional efficiency was key to ensuring high titers over time, post-translational bottlenecks were identified as the primary limitation to cellular productivity (qP), with mAb production systems at the time achieving a maximum of 20–50 pg/cell/day (64). mAb titer was found to be correlated with mRNA expression up to a threshold point beyond which increases in mRNA did not result in increased expression (74). Therefore, most engineering efforts to increase qP focused on alleviating bottlenecks in assembly and folding reactions through overexpression of chaperones. Unfortunately, many studies performed during this time showed mixed results, with benefits appearing to be cell and protein specific, so solutions for improving culture productivity (titer) attempted to balance cell growth and qP to obtain optimal results but rarely improved qP significantly (64).

Upstream Process Development: Increasing Culture Scale and Titer

To meet the increased demand for recombinant therapeutics, many of the early, low-titer processes for mAbs required bioreactor capacities of greater than 10,000 L, causing manufacturers to invest heavily in increasing production capacity. Large-scale manufacturing facilities were constructed throughout the 2000s, including facilities with bioreactor sizes ranging from 10,000 L to 25,000 L and total capacities up to 200,000 L (75). By 2009, manufacturers were routinely achieving mAb titers of 1–5 g/L from 7–14-day fed-batch processes (76). The general approach taken to achieve these titers was to rapidly reach a high viable cell density (VCD) for maximum productivity and then maintain the culture at that productive VCD for as long as possible (64). Optimization of media and feeding strategies, plus careful consideration of scale-up parameters to ensure constant cellular environments across scales, helped cultures maintain high productivity even at larger scales.

Bioreactor processes generally controlled key parameters such as temperature, pH, dissolved oxygen, dissolved CO_2 (dCO_2), mixing, and nutrient levels (through feeding strategies) while measuring cell concentration, viability, and metabolites to monitor culture health (77). Dynamic feeding strategies designed to control key nutrients at an optimal setpoint demonstrated that maintaining a favorable metabolite profile throughout culture can improve both productivity and product quality (78). With concerns about shear sensitivity largely abated with the use of Pluronic-F68, appropriate scaling of mixing and aeration parameters was found to be crucial to ensure adequate oxygen transfer rates and CO_2 removal (79). A comparison of dCO_2 levels in bench-scale (1.5 L) and pilot-scale (1,000 L) cultures showed the difficulty in removing CO_2 in large reactors owing to a reduced surface-to-volume ratio (80), and subsequent studies in scale-down models showed that bioreactor scaling based on a constant power per volume gave an acceptable dCO_2 profile at the 2-L scale (81).

Improvements to cell culture media throughout this time yielded formulations better suited to providing optimal nutrient levels throughout culture. Media moved from serum free to completely chemically defined, with the quantities of all components (sugars, amino acids, vitamins, trace minerals, salts, lipids, and proteins) known and systematically determined (77, 82). Determining the optimal concentrations of each component, however, was time consuming

and expensive if each concentration was evaluated individually, leading to the use of rational design methods to find media formulations that optimized metabolic efficiency while minimizing toxic waste product formation (44, 83). Approaches included developing stoichiometric models for basal and feed medium composition using cell growth and metabolite consumption rates (84), substituting key metabolites to modulate byproduct formation (85), and using design of experiments (DOE) to efficiently identify media components with the strongest impact on culture performance (86). Improved media formulations also allowed manufacturers to develop host cell lines capable of serum-free, suspension growth from which producer cell lines could be derived, instead of adapting each producer cell line individually, which reduced PD timelines and allowed for standardization of cell culture processes (42, 87, 88).

Batch: a cell culture process where cells are provided nutrients at the beginning of the process with no further supplementation

Downstream Process Development: Establishment of Platform Processes

Advances in downstream processing technology focused on increasing throughput and ensuring purity to accommodate the large batch sizes, high titers, and stringent purity criteria for biopharma products. Because chromatography resin capacity is based on the mass of product to be purified rather than the volume to be processed, high-titer mAb processes created bottlenecks in purification unit operations (89). Resins available in the 1990s were unable to process batches reaching 2–5 g/L, yet significant improvements in binding capacity during the 2000s allowed purification of batches up to 5 g/L by 2009 (76).

The downstream process plays a crucial role in determining final product purity by removing process impurities [residual host cell proteins (HCPs), DNA, ligands, and additives], controlling product quality (aggregates, charge variants), and clearing virus. With the increase in the number of mAb products coming through company pipelines, and the growing body of knowledge about how purification unit operations impacted these product quality attributes, manufacturers created platform downstream processes to increase the speed with which mAb candidates could be brought to the clinic (**Figure 2**). These templated approaches were enabled by the standardization

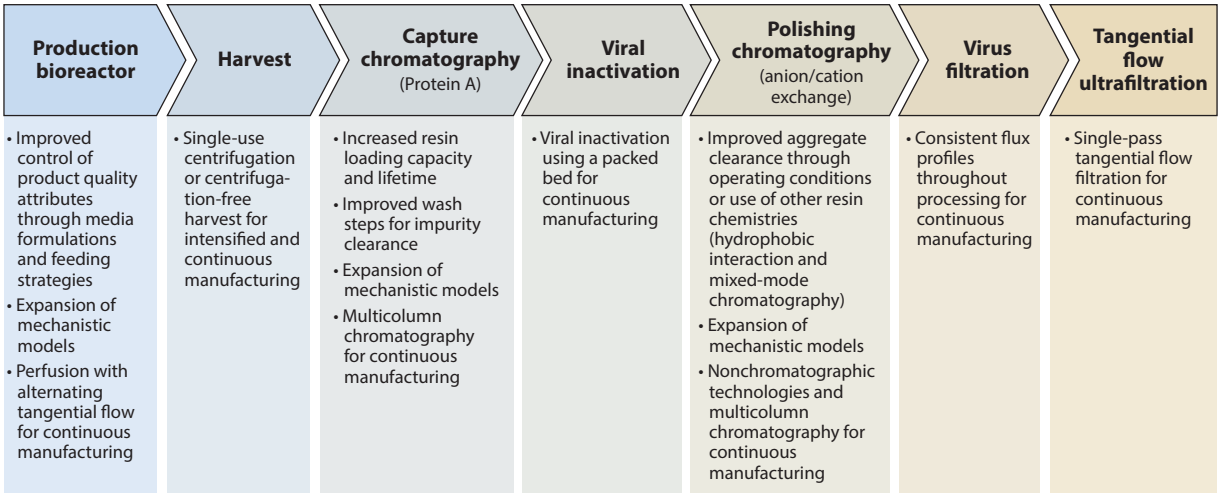

Figure 2

A consensus monoclonal antibody (mAb) platform manufacturing process to produce drug substance with areas of active research included below each unit operation. Prior to the production bioreactor, cell banks are thawed and expanded through the seed train up to the inoculum (N-1) bioreactor.

of mAb framework sequences, and they leveraged PD and scale-up knowledge across products (90). A platform process typically included a defined set of unit operations and the parameter ranges within which they could be operated, providing a common starting point for new products entering PD. Narrowing the range of operating conditions helped to limit the scope of experimentation needed to define a purification process with acceptable yields and purity levels for a new mAb. By 2009, many companies converged on a consensus mAb production process: cell culture in a stirred tank production bioreactor followed by harvest, Protein A affinity chromatography, viral inactivation (VI), one or two IEX chromatography steps (anion and/or cation exchange), virus filtration, and tangential flow ultrafiltration (TFUF) to produce drug substance (76, 90, 91).

Despite early doubts about the cost and efficiency of Protein A resins, Protein A's specificity led to its widespread adoption in platforms (89). Mutation of alkaline-sensitive asparagine residues in the Protein A sequence improved ligand stability for sodium hydroxide–based clean-in-place operations (92), with column lifetimes of up to 300 cycles reported (51). Improvements to resin matrix chemistry and ligand densities increased dynamic binding capacities to greater than 30 mg mAb/mL resin, exceeding previously reported capacities of less than 20 mg mAb/mL on older resins (60, 89, 93). To address the high cost of Protein A resins, companies adopted cycling methods for increasing resin use, emphasizing the importance of column lifetime (91, 94). Whereas IEX chromatography was recommended for polishing steps in most processes, hydrophobic interaction chromatography and mixed-mode chromatography were investigated as IEX alternatives for products with specific aggregate clearance challenges (94). Platforms also adopted stringent virus clearance strategies using orthogonal removal methods such as low-pH inactivation, parvoviral-grade virus filters (~20-nm pore size), membrane absorbers, and various chromatography modes (95).

Impact of Regulation Reforms on Process Development

In conjunction with the increase in production scale and the number of products in company pipelines came increased challenges in identifying and addressing the root causes of problems that arose during manufacturing processes, leading to batch failures and product waste (96). At the same time, changes in the FDA's approach to regulation created an enormous burden on regulators to keep up with new product applications and supplements for process changes. To address these issues, the FDA launched the Pharmaceutical cGMP Initiative for the 21st Century in 2002, followed by guidance on Process Analytical Technology (PAT) in 2004 (97) and adoption of the International Conference on Harmonization's guidance on Quality by Design (QbD) in 2006 (98). The new QbD paradigm, and by extension PAT, focused on the design and understanding of processes to consistently produce a product that meets a predefined quality profile (99). Prior to QbD's introduction, many processes were left to operate at the chosen conditions, and manufacturers accepted the resulting product quality profile. In contrast, QbD aimed to design quality into the manufacturing process from the start by using the target quality profile as a guide for process design, ensuring that manufacturers could consistently and reliably produce high-quality product.

The QbD workflow identifies critical quality attributes (CQAs), characteristics that the product should possess to ensure clinical performance, and critical process parameters, parameters that must be kept within specified ranges to consistently produce a product with the desired CQAs. PAT focuses on implementing new technologies for "timely measurements" of critical quality, performance, and process attributes and plays a complementary role to QbD to enable real-time measurements and dynamic responses to deviations (89). Where early processes relied on offline measurements (performed on removed samples in quality-control labs) or at-line measurements

(performed on removed samples in proximity to the process) taken infrequently during processing, PAT encouraged a shift to a combination of at-line, on-line (performed on diverted in-process material), and in-line (performed directly on in-process material) measurements for continued monitoring of process performance (100). Several new technologies spanning all three types were introduced during this time for monitoring of CQAs and critical process parameters (100).

QbD's introduction of the design space, a multidimensional space of operating conditions within which all process parameters can operate and still generate high-quality product, necessitated a shift toward risk management and increased process understanding to determine the connections between operating conditions and product quality, creating a new set of research focuses within PD groups. Rational multivariate study approaches such as DOE or in silico modeling during PD emerged as the preferred methods to efficiently capture main effects and interactions between process parameters using minimal runs (101). To put these new guidelines into context and to demonstrate how QbD could be applied to real-world products, an industry-wide working group published the A-Mab case study in 2009 as a broadly applicable example of the use of QbD principles for product and process development (102).

Omics: an umbrella term for systems biology fields that perform comprehensive assessments of different types of biological molecules

BIOPHARMA TODAY: NEXT-GENERATION PLATFORMS AND CURRENT CHALLENGES (2010–PRESENT)

Biopharmaceutical approvals from 2010 onward have showed no indication of slowing down, with a record 112 approvals between 2015 and July 2018. Of those approvals, more than 50% were mAbs, and starting in 2012, mAbs consisted of more than 50% of each year's total global sale of biopharmaceuticals (2). The growth of systems biology has driven many of the major cell line engineering advances in the last ten years, with research focused on manipulating cellular behavior with unprecedented control. Upstream and downstream process technology, however, has largely reached a point of maturity, with many of the same unit operations from the late 2000s still in use today. To reflect the establishment and growth of QbD initiatives, major research emphasis has been placed instead on understanding the connections between existing process operations and product quality.

Cell Line Development: Application of Systems Biology for Genome Engineering

The emergence of systems biology tools and omics technologies has provided the biopharma community with large, comprehensive data sets that quantify the differences in cellular functions between CHO cell lines and the changes they undergo throughout the cell line development process (103). The publication of the ancestral CHO-K1 genome in 2011 provided the first publicly available annotated genome and gene expression data set to support genome engineering efforts (104). Subsequent sequencing of the *C. griseus* genome and comparison to multiple derivative CHO cell lines showed the extent of the genetic rearrangements and mutations that CHO had undergone since its isolation from hamster (105). Improvements to the *C. griseus* genome increased sequence quality and continuity with the PICR assembly and achieved chromosome-scale scaffolds in the PICRH assembly (106, 107). Application of other omics technologies, such as epigenomics (108), transcriptomics (104, 109), proteomics (110, 111), and metabolomics (83), has generated CHO-specific information about cellular pathways related to growth, metabolism, protein production, and posttranslational modifications, as well as responses associated with common PD processes including adaptation, extended culture, and cloning. Recent cell line engineering work has used information from omics data sets to identify gene targets across many

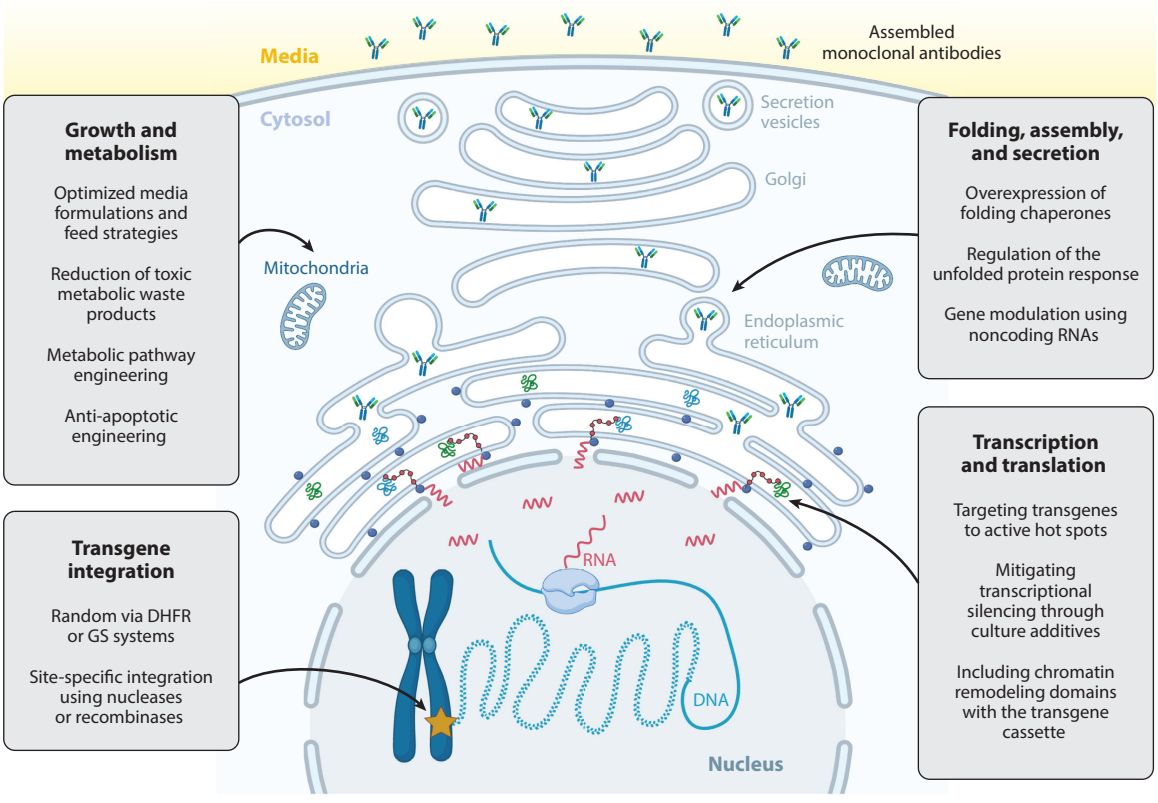

Figure 3

Cell line engineering efforts to improve cellular and culture productivity have targeted each step in the protein production process and have been aided by the recent expansion of available CHO-specific omics data sets. Abbreviations: DHFR, dihydrofolate reductase; GS, glutamine synthase. Figure adapted from images created with BioRender.com.

cellular functions for overexpression, knockout, or regulation by noncoding RNAs to improve protein production (112–114) (**Figure 3**). Curation of multiple omics data sets into a consensus genome-scale model for CHO metabolism has allowed researchers to investigate metabolic perturbations and assess their impact on cell growth and protein production in silico (115).

With the major drivers of production instability identified with the help of systems biology tools, work continues to better understand the underlying molecular mechanisms and find targeted engineering solutions. Studies in producer CHO cell lines, particularly those generated through gene amplification methods, verified that gene silencing resulted primarily from epigenetic changes such as promoter methylation and histone deacetylation (116, 117), and copy number loss was driven by genetic rearrangements and deletions (118). It has also been suggested that deficiencies in the DNA double-strand-break repair machinery of CHO cell lines contribute to the observed genomic instability, and restoration of these pathways may improve production stability (119). Yet in the absence of universal criteria for early identification of stable-producing clones, developing SSI platforms to integrate transgenes at known epigenetically stable and transcriptionally active genomic loci (referred to as hot spots) has become the solution of choice for mitigating production instability (120). Identification of hot spots has been crucial for SSI

implementation, with most published hot spots identified through random genetic screens (121, 122). Some hot spots have also been granted patent protection, demonstrating their value to the biopharma industry (123, 124). However, hot spot identification has been limited by difficulties in rationally predicting their location as genomic and epigenomic contexts defining a hot spot may vary across cell lines (120). A recent study found that 10.9% of the CHO genome maintained favorable and stable epigenetic and transcriptional profiles across varying conditions, suggesting that these stable regions of the genome may be good targets for finding novel hot spots (125).

SSI platforms can be built using nuclease-mediated, recombinase-mediated, or hybrid approaches. Nuclease-mediated systems involve the direct integration of the transgene of interest at a targeted locus using a programmable endonuclease (such as zinc finger nucleases, transcription activator–like effector nucleases, or CRISPR/Cas9) (126, 127). These mechanisms for transgene insertion, although capable of precise targeting, remain hindered by their reliance on endogenous but inefficient HDR mechanisms in CHO, with studies reporting absolute SSI efficiencies using CRISPR/Cas9 around 1–2% (128, 129). Recombinase-mediated approaches rely on the integration of a landing pad, containing recombinase recognition sites, reporter, and selection genes that can later be exchanged through RMCE for the transgene of interest. Many systems using this approach, however, have integrated the landing pad into an unknown genomic location, requiring time-intensive cell line screening (121, 130). Alternatively, hybrid approaches seek to use targeted integration to insert landing pads capable of RMCE into a known hot spot, eliminating the need to screen for high-expressing cell lines, yet these approaches require the establishment of a platform cell line containing the landing pad prior to use (131, 132). Despite the promises of these platforms to mitigate production instability, SSI systems rely on expression from a single copy of the transgene of interest, leading to lower expression levels as compared to random integration cell lines. The optimization of transgene cassettes for increased expression from these systems is an active area of research (132, 133).

Although cellular productivities have continued to improve, with recent cell lines reaching 50–90 pg/cell/day, increased reports of difficult-to-express proteins hindered by posttranscriptional bottlenecks have spurred continued efforts to increase cell secretory capacity through cell line engineering (134–138). Systematic studies and modeling across cell lines (139) and mAb products (140) showed that both the product and the host cell will impose certain limitations on expression, and solutions tailored to the specific nature of each bottleneck will be most effective in increasing qP. Most engineering approaches have attempted to regulate targeted chaperones and proteins involved in the unfolded protein response, because unfolded protein response activation from endoplasmic reticulum stress has been observed as a hallmark of bottlenecked cells (134, 141). As was observed previously, few effector genes have been found that broadly increase expression of recombinant proteins, and successful engineering solutions have been context specific; however, genome-scale modeling of secretory gene overexpression showed that this approach holds greater potential for increasing productivity as compared to bioprocess treatments (115).

Upstream and Downstream Process Development: Maturation of an Industry

The continued use of platform processes has established a mature approach to PD and manufacturing of new therapeutics. Current biopharma development work has been viewed as "evolutionary" instead of "revolutionary" (76), with focus placed on refinements to current operations and enhanced process understanding to ensure product quality and process consistency. Incremental improvements to media formulations, feed strategies, and cell lines have pushed mAb titers to 8 g/L, which has been cited as an upper limit for titer beyond which limited process or economic improvements would be made (142), with some processes demonstrating mAb titers of >10 g/L

(143). With sufficiently high titers and downstream capacities, cost of goods may no longer need to be a key driver of biopharmaceutical price or process design (76, 144). With QbD as the primary motivator instead, significant effort has been put toward high-throughput data collection, mechanistic understanding, and process modeling, with two major goals: (*a*) to improve experimental and computational models to better predict large-scale process outcomes with reduced development efforts and (*b*) to connect process parameters to their impact on product quality attributes.

New experimental methods take advantage of advances in automation and throughput. Automated small-scale bioreactors, such as the Ambr® system (Sartorius), are now an important tool in PD labs to assess many different operating conditions in a lab-scale format that is more representative of larger-scale operations (145). Miniaturized chromatography methods amenable to automation via liquid handlers were developed previously for assessing resin binding capacity (146) and have since been extended for evaluation of chromatographic operating conditions (147). Although deviations between miniature and lab-scale columns have been observed, mechanistic modeling of the differences between scales has improved predictions (148). Both technologies offer increased throughput for more rapid screening of conditions and are used frequently for DOE-based studies.

Bioreactor complexity and the dynamic interplay between process conditions and cellular metabolism have made mechanistic modeling of bioreactors difficult. Computational fluid dynamics has been used to model fluid flow in stirred vessels and to determine mixing times but has yet to be meaningfully integrated with metabolic information (149). Information derived from systems biology has largely driven the models available for cellular processes, as discussed above. Prediction of upstream process outcomes and identification of key process parameters have relied on multivariate statistical analyses using data mined from production-scale runs, without other mechanistic information (150). DOE studies have identified statistical relationships between process parameters and product quality data that could be used as the basis for dynamic control strategies, especially for critical attributes like glycosylation for which mechanistic understanding also exists (151, 152). However, in the absence of real-time product quality measurements, the application of these relationships may be limited.

Mechanistic models are more prevalent for downstream unit operations, where mathematical descriptors of flow, mass transport, and adsorption isotherms are available (149). However, fully accounting for the complexities of biopharmaceutical products in these models has posed new challenges, as molecular-level information about protein–ligand interactions and the structural changes that proteins undergo during different processing modes is limited. Regardless, mechanistic models using various assumptions for flow and isotherm behavior have routinely been used for the optimization of processing conditions across many different chromatography modes (153). Downstream development has also benefitted from the increase in omics methods to understand the interactions between processing and product quality. Following the identification of retained HCPs with negative impacts on product quality (154, 155), proteomics tools were used to study HCP retention mechanisms (156, 157) and proteome changes with culture age (111) to identify dozens of novel, difficult-to-remove HCPs, supporting efforts for improved HCP clearance.

PAT initiatives have encouraged the transition to continuous parameter monitoring, especially for bioreactors, with the goal of collecting real-time data that can be used to make decisions about the process to ensure quality. Spectroscopic methods such as Raman, Fourier transform infrared, and near-infrared spectroscopy have all shown promise in continuous measurement of culture metabolites; capacitance sensors have been evaluated for online cell-density measurements; and mass spectrometry has been used to monitor cell stress (158). Downstream processes have historically employed PAT to measure certain process parameters, such as pH or conductivity, so many technologies explored for downstream PAT focus on expanding to other parameters (such as

protein concentration) or real-time monitoring of product quality attributes. High-performance liquid chromatography is of particular interest for measuring product quality, especially aggregation, during downstream processing steps; however, implementation of PAT for downstream operations is more challenging given the short time window during processing in which decisions must be made (158).

EMERGING CONSIDERATIONS: THE FUTURE OF BIOPHARMA

Future research topics in the biopharma industry will likely focus on increases in flexibility, speed, and control to address changing economic demands and increase accessibility to medicines. With the emergence of more diverse product portfolios and personalized therapies with smaller patient groups, manufacturers are moving away from the large manufacturing suites seen in the early 2000s in favor of smaller, more flexible spaces that can rapidly transition between products and potentially accommodate the intensified processes currently under evaluation. Increasing production speed through flexibility instead of size also gives manufacturers the ability to respond quickly to changing global conditions, ensuring supply chain integrity and therapeutic availability. Alongside these types of process advancements, however, comes an increase in process complexity, necessitating investments in process control technologies to maintain the high standards of product quality achieved currently and adhere to established QbD initiatives.

New Therapeutic Modalities

PD for next-generation mAb modalities, including bispecifics and antibody–drug conjugates, and viral vectors for C&GTs will require increasing attention as these products enter the market in higher numbers. The concept of a bispecific antibody was first described in the 1960s, and more than 100 different formats have since been developed (159). The complexity of bispecific formats, however, requires these products to undergo additional engineering at multiple points during PD to optimize the production of correctly assembled products, and solutions can vary from one framework to another (159, 160). Recent antibody–drug conjugate development has focused on the chemical linkage steps required and subsequent purification (160). Manufacturing processes for C&GT viral vectors have some parallels to those used for recombinant protein production, and although the upstream hosts are typically insect cells or human embryonic kidney cells rather than CHO cells, early vector processes have leveraged existing knowledge and capabilities. Similarly, downstream purification of viral vectors relies on IEX chromatography, a well-established unit operation in recombinant protein processes (161, 162).

Continuous Manufacturing

In an effort to increase processing flexibility, reduce development time, and better respond to fluctuating market demands, continuous manufacturing strategies have gained interest as possible ways to reduce facility costs, ensure reliable drug supplies, and improve process consistency (163–165). Historically, technological limitations and concerns over process complexity and failure rates have hindered the full implementation of end-to-end continuous manufacturing at the commercial scale. However, recent advances in technology have improved the feasibility of large-scale implementation. Current technologies primarily under consideration include perfusion cell culture using alternating tangential flow (ATF), multicolumn chromatography methods, continuous VI, and single-pass tangential flow filtration (SPTFF), many of which could also be implemented as stand-alone single-use systems.

Continuous upstream processing had been adopted in the biopharma industry on a limited basis as early as 1994 and was used most frequently for labile recombinant blood factors and enzymes

that demonstrated limited stability in culture (166). Early uses of perfusion for mAb production were hampered by the high fouling rates of cell retention devices at high cell densities that could contribute to culture failure. The development of ATF technology, in which cell culture fluid is pumped in alternating directions across a hollow-fiber membrane, addressed these shortcomings by reducing shear damage and minimizing fouling. VCDs of greater than 100×10^6 cells/mL have been maintained for extended periods in ATF systems (167). ATF perfusion systems were first successfully integrated with downstream processing steps in 2012 (163) and have since demonstrated increases in productivity (167), a narrower range of cellular residence time, and more consistent product quality profiles (144, 168). Efforts to model the economic benefits of perfusion cell culture have found that perfusion rate, media formulation, titer, and process scale all contribute to process performance, and optimization of multiple parameters, which may differ between companies and products, may be necessary for perfusion systems to be beneficial (166, 169).

Although several new technologies for continuous downstream processing have been introduced in recent years, a continuous downstream process has yet to be implemented at the commercial scale (144, 164). The use of periodic countercurrent chromatography (PCC) for capture, in which multiple chromatography columns are operated in series with the breakthrough of one column captured on the next, is of particular interest. Each column is then cycled through wash, elution, and cleaning/regeneration steps. Whereas in traditional chromatography the full capacity of the resin is not used, PCC allows for increased loading of each column, which increases resin use, decreases column sizes, and reduces buffer usage (144). However, the complexities in PCC control and automation and need for PAT have limited its use in commercial manufacturing. As the name implies, PCC is technically periodic and not truly continuous, so integration of PCC capture methods into an end-to-end continuous process with a VI step following capture has been challenging. Complete inactivation of virus requires product incubation at a low pH, which is easily controlled in a batch format. However, a continuous VI step must not only accommodate the pH gradients present in the elution from the capture step but also ensure a minimum residence time. To address these requirements, continuous VI using flow through a packed bed has been suggested with the incorporation of a hold tank upstream to minimize any pH gradients and control flow rate if needed (167, 170).

The development of continuous options for TFUF, such as SPTFF, has contributed to advances in truly end-to-end continuous processes. Traditional TFUF for concentration and buffer exchange in batch mode involves recirculating product through the filter and a feed tank until the desired concentration and buffer composition have been reached, requiring many passes through the filter to achieve the target values. Conversely, SPTFF units contain multiple conventional tangential flow filters in one device, with sets of parallel filters operated in series and the feed making only one pass through the filters (171). SPTFF technology has been demonstrated both for volume reduction of in-process feed streams (167, 171) and for concentration of product to typical drug substance values (172). Operation of multiple SPTFF units in series with subsequent buffer dilutions has been demonstrated for continuous diafiltration, achieving greater than 99.75% buffer exchange with a three-stage SPTFF design (173).

Although the promise of highly productive, continuous processes is intriguing, their true economic benefits have yet to be determined, and economic feasibility may be company, process, and product specific (169). Kelley et al. (142) argue that the complexity of automating, monitoring, and troubleshooting a continuous process is significant, and the low cost of goods at which products can be made with current platforms may not support the investment in continuous-enabled facilities. Even with the higher productivities achieved via continuous processes, concomitant increases in other costs (consumables or reagents) sometimes outweigh the benefits, depending on scale (169). However, reductions in process footprint leading to increased domestic production

capabilities may allow for more agile responses to drug shortages and ensure a reliable, high-quality supply of medicines (164). The business risks associated with regulatory acceptance must be considered and addressed before commercial biopharma products produced from continuous processes are likely to come to market (174).

Pandemic Response

The ability of the biopharma industry to rapidly deploy mAb manufacturing processes was demonstrated in response to the COVID-19 (coronavirus disease 2019) global pandemic (3). At the pandemic's outset, several companies made significant and substantial investments to develop mAbs on highly accelerated timelines to bring much-needed medical countermeasures to a society in need, while other technologies, such as mRNA vaccines, were being developed in parallel. The decision to invest resources, and accept relevant business risks, ultimately proved successful with Emergency Use Authorization granted to Regeneron's REGEN-COV2 (casirivimab + imdevimab) and Lilly's bamlanivimab less than one year after the public health emergency was declared (3). However, in January 2022, those authorizations were withdrawn with the emergence of new virus variants that limited the effectiveness of these products, which consequently significantly reduced the potential return on those early investments (175). With the development capabilities of mRNA vaccines now demonstrated, mRNA technology has emerged as a platform approach to vaccine manufacturing that will surely be deployed in response to future pandemics. Given the current state of today's mAb manufacturing technology, it may no longer be in the interest of companies to rapidly develop mAbs under these circumstances given the scale of investment and risk of potentially limited return, in light of how rapidly vaccines might be developed. However, the advent of new approaches (such as continuous manufacturing or SSI) may provide opportunities for mAb manufacturers to rapidly respond to future public health emergencies with less expense and risk.

CONCLUSIONS

Technological maturity has been achieved in the biopharma industry through decades of cross-disciplinary scientific innovation and collaboration. Modern processes capable of consistent production of high-quality, complex biopharmaceutical products were made possible by foundational knowledge established by manufacturers of early biological products and scientific discoveries from academic and industry researchers in fields such as chemical engineering, biology, chemistry, bioinformatics, and statistics. What early processes lacked in technological advancement, they remedied with creative engineering solutions. The unit operations used were varied, complex, and challenging to scale up; however, manufacturers still successfully implemented these technologies into processes routinely to generate therapeutics at scales required to meet increasing market demand. The evolution of processing technology and increased communication with regulators have resulted in a transition to product-focused approaches which motivate current research on understanding relationships between processing conditions and product quality. The eventual convergence of mAb manufacturing processes to a common set of unit operations suggests that mAb processing has reached an optimal point and that a revolutionary advance in process technology would be required to disrupt the status quo. The knowledge gained from traditional mAb processing has supported the development of novel therapeutic modalities, evidenced by the increased prevalence of these therapies in the clinic. Continuing to leverage industry knowledge and collaborations between academics, industry, and the government can drive innovation to increase manufacturing capacity, ensure reliable supply chains, and improve the cost of medicines. Looking forward, the industry will continue accelerating the development and deployment of critical lifesaving and life-altering medicines and vaccines to patients for decades to come.

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