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Current PAT Landscape in the Downstream Processing of Biopharmaceuticals

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ABSTRACT

Protein-based therapeutics have revolutionized modern medicine, addressing complex diseases with unprecedented specificity and efficacy. The rising demand for biologics has driven the evolution of biomanufacturing practices to ensure consistent quality and operational efficiency. Traditional batch testing, with its inherent limitations, is being replaced by quality by design (QbD) frameworks and process analytical technology (PAT). PAT facilitates real-time monitoring and control by integrating advanced analytical tools and data-driven methodologies to optimize downstream processing (DSP). This review highlights the recent advancements in PAT tools, including spectroscopy, chromatography and biosensors. Spectroscopic techniques provide rapid, non-invasive measurements, while biosensors offer high specificity for monitoring critical quality attributes. Additionally, the integration of chemometric modelling and digital twins enables predictive analytics and enhances process control, paving the way for real-time release (RTR) of the product. Despite challenges in regulatory compliance and technology integration, innovations in automation and machine learning are bridging these gaps, accelerating the transition to intelligent manufacturing systems. This article provides a comprehensive evaluation of emerging analytical technologies and strategic insights into their integration, aiming to support the biopharmaceutical industry's shift towards robust, continuous and adaptive manufacturing paradigms.

1 | Introduction

Protein-based therapeutics are highly successful and constitute around half of the top-selling drugs in the market. Proteins are involved in molecular signalling, transportation, maintenance of cellular and tissue integrity, immune function and many more. Therefore, protein-based therapeutics have been developed to treat problems associated with any of these functions. Common types of protein therapeutics include antibodies, enzymes, coagulation factors, hormones and cytokines [1].

Protein therapeutics, often referred to as biopharmaceuticals or biologics, have inherent advantages over small molecule drugs as they are highly specific and have complex functions in the body. As a result, they tend to cause less interference with normal biological processes and fewer adverse effects [2]. These proteins can either be purified from their natural sources or can be mass-produced in suitable host cells using recombinant DNA technology [2]. An example of proteins purified from their native sources are plasma-derived therapeutics, which include antibodies, clotting factors, etc. [3].

Abbreviations: ADC, antibody-drug conjugates; ATF, alternating tangential flow; CNN, convolutional neural network; CPP, critical process parameter; CQA, critical quality attribute; DoE, design of experiments; DSP, downstream processing; IR, infrared spectroscopy; LSPR, localized surface plasmon resonance; MALS, multi-angle light scattering; MIR, mid-infrared spectroscopy; NIR, near-infrared spectroscopy; PAT, process analytical technology; PCC, periodic countercurrent chromatography; PLS, partial least square; QbD, quality by design; QbT, quality by testing; RTR, real-time release; SEIRA, surface-enhanced infrared absorption spectroscopy; SERS, surface-enhanced Raman spectroscopy; SMB, simulated moving bed chromatography; SPTFF, single pass tangential flow filtration; TPP, target product profile; UHPLC, ultra-high performance liquid chromatography; UPLC, ultra-performance liquid chromatography; UV-Vis, ultraviolet visible; VPE, variable pathlength slope; WGM, whispering gallery mode.

The first use of recombinant DNA technology was in the production of insulin from host cells (*E. coli*) in the 1980s [4]. Since then, the biopharmaceutical industry has experienced tremendous growth, and now includes a portfolio of novel biologics including monoclonal antibodies [5], bio-specific antibodies [6], fusion proteins [7] and antibody drug conjugates (ADCs) [8]. As of 2023, more than 350 protein therapeutics have been approved by FDA [1]. These innovative therapeutics offer treatment options for patients with debilitating conditions that were previously untreatable, thereby having huge demand, growth and market share within the overall pharmaceutical market. Monoclonal antibodies are the highest selling drugs among protein therapeutics, and some examples of the top sellers in the market include Humira (adalimumab) from AbbVie to treat multiple autoimmune pathologies, Revlimid (lenalidomide) from Celgene used to treat multiple myeloma and Opdivo (nivolumab) from Bristol-Myers-Squibb as an immunotherapy for many types of cancer [9].

With demands for biologics growing exponentially, there is also a rising need to ensure the efficacy and safety of these drug products, necessitating efficient quality assessment and control systems in place. Traditionally, batch testing has been performed to ensure the quality of the drug products, where the manufactured drugs are selected at random for every batch for testing. This has two main disadvantages: (i) the quality is only known after the manufacture of the drug, leaving very limited scope for corrective actions, potentially leading to rejected or failed batches and (ii) this approach assumes that all the units in a batch are uniform without any variability, and the decision for product release is made based on the analysis of one representative unit for every batch [10]. This increases the risk of the distribution of poor-quality drugs. In fact, the pharmaceutical industry spends up to 20% annually on handling defective products, which is equal to 20% of annual sales [11], placing a huge financial burden. This challenge is especially important given the industry's substantial growth, with global revenues reaching approximately $1.6 trillion in 2023 [12].

The drawbacks of the quality by testing (QbT) approach are even more pronounced for biopharmaceuticals, as they are more complex and involve therapeutic proteins being 50–1000 times larger than small molecule drugs. Unlike small molecules, which are produced through chemical synthesis, most biopharmaceuticals are produced via biosynthesis, using genetically modified organisms [10].

Recombinant proteins are produced using various expression systems, such as bacteria, yeast, insect and mammalian cells [2], in a process known as upstream processing. This process begins with the selection of suitable host cells to express the target protein, followed by the propagation of cell lines through stages—starting in shake flasks and seed bioreactors before final transfer to a production bioreactor. Depending on the host organism, the target protein is produced intra- or extracellulary [13]. This is followed by downstream processing, to extract and purify the final product, encompassing multiple steps such as product recovery from the culture medium, virus inactivation, protein capture and sequential purification using various techniques [14].

The inherent complexity of the manufacturing process and little control over the biosynthesis as compared to the chemical synthesis of small molecules [10] necessitate a thorough understanding of the process and a more sophisticated approach to ensuring the quality of biopharmaceuticals. The International Council for Harmonization of Technical Requirements for Pharmaceuticals for Human Use (ICH) introduced quality by design (QbD), defining it as '*A systematic approach to development that begins with predefined objectives and emphasizes product and process understanding and process control, based on sound science and quality risk management*' [15].

The implementation of QbD (Figure 1) starts with defining the quality target product profile (qTPP) for the final product. According to ICH, the qTPP is defined as '*A prospective summary of the quality characteristics of a drug product that ideally will be achieved to ensure desired quality, taking into account safety and efficacy of the drug product*' [15]. Defining the qTPP forms the basis for listing all the potential critical quality attributes (CQA), which are either physical, chemical or biological properties that must remain within a specified range or limit to ensure the qTPP. Certain process parameters, known as critical process parameters (CPP), have a variability that impacts the CQAs and therefore must be monitored or controlled [15].

QbD involves (i) precise identification of CPPs and CQAs, and (ii) designing the processes to deliver these attributes for achieving qTPP. Essentially, QbD aims at establishing a 'design space' where the quality is built into the process, in contrast to measuring the product quality at the end [16]. This needs an improved understanding of the processes and the impact of process parameters and product attributes on the final product.

The 'design space' for processes can be defined by performing process characterization studies using the design of experiments (DoE) approach. This method relates the CQAs to process variables and helps understand the effects of different factors and their interactions. Based on this understanding, multidimensional models are built to link CQAs to various factors, enabling the definition of acceptable ranges for process parameters. A control strategy is then created to ensure that the process meets predefined specifications [17].

The control strategy can be employed by using process analytical technology (PAT), a key driver for QbD implementation. PAT provides the platform for continuous and real-time monitoring of biopharmaceuticals during the production process, enabling in-process control. FDA defines PAT as '*a system for designing, analyzing, and controlling manufacturing through timely measurements (i.e., during processing) of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality*' [18].

The key goal is the integration of analytical technologies in-line, on-line or at-line (Figure 2) with manufacturing equipment, for process monitoring and control [19]. PAT is a term that encompasses a broad spectrum ranging from measurement systems, control strategies and analysis of data to enable continuous monitoring and process control. This system is essential to implement QbD and ensure real-time release (RTR) of the product [19].

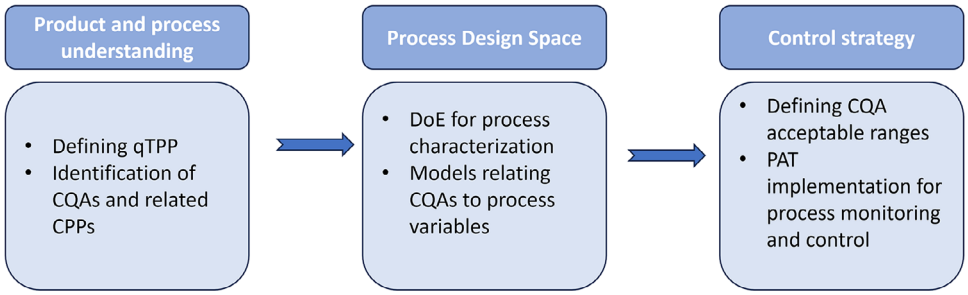

FIGURE 1 | Overview of steps involved in QbD implementation.

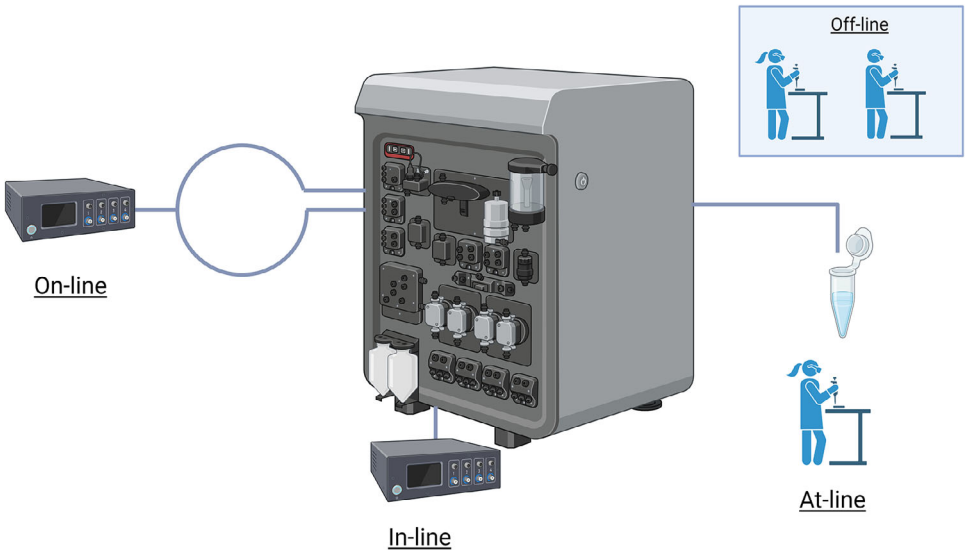

FIGURE 2 | An illustration of in-line, on-line, at-line and offline setups of analytical tools for bioprocess monitoring. For in-line analysis, the analytical tool is directly integrated into the process stream, allowing continuous, real-time measurement without sample removal. For on-line analysis, a portion of the process stream is diverted to an analyser, where measurements are conducted in real time or near real time, with the sample typically returned to the process. For at-line analysis, samples are manually extracted from the process and analysed immediately near the production line. In contrast, offline analysis involves samples being collected from the process and analysed at a remote laboratory or location, typically resulting in longer delays between sampling and obtaining results.

Given that several analytical techniques have been explored as promising PAT tools, this review will focus on process analytical technology tools applied to downstream processing.

2 | Advancements in Downstream Operations and Process Control

In biomanufacturing, while the upstream process is dedicated to optimizing conditions for cell cultivation and target molecule production, the downstream process focuses on extracting and purifying the desired molecules from the resulting biomass using a combination of chromatographic, filtration and centrifugation techniques [14].

Downstream processing (DSP) begins with the harvesting and clarification of the target product from the upstream fermentation or cell culture broth, followed by an initial capture step, such as Protein A chromatography for antibodies, which concentrates the target molecule while reducing impurities [20]. Subsequent purification steps employ a range of chromatographic techniques tailored to the product's properties, including ion exchange, size exclusion, or affinity chromatography, to achieve the required purity and quality [21]. The final stage often involves ultrafiltration/diafiltration (UF/DF) to formulate the product with stabilizing excipients to ensure its long-term stability and suitability for therapeutic use [22].

DSP constitutes a pivotal and crucial phase in the production process, as it involves the purification of the target molecule, directly impacting the final product quality [14]. Purification often exceeds the cost of upstream manufacturing, with downstream processing accounting for 80% of production expenses, largely driven by the high cost of Protein A resin and virus filtration [14, 23]. Therefore, it has become imperative to optimize bioprocesses that can deliver high-quality products more efficiently with cost advantages.

This need has led to a shift in focus of biomanufacturing from traditional batch-wise processing towards process intensification and continuous manufacturing, where all the unit operations are

integrated seamlessly to create a single flow, with no or minimal hold volumes in between [24]. In upstream processing, perfusion bioreactors were developed to continuously remove spent culture media containing the target product, maintaining optimal cell densities within the bioreactor for better process control. This approach had led to higher cell densities thereby increasing bioreactor productivity [25]. While such advancements had been made and were well established for upstream processing, integration of continuous downstream processing is still evolving [26].

Efforts had been made to develop a continuous manufacturing process for downstream processing. This includes the use of cell separation devices such as alternating tangential flow (ATF) [27] and single pass tangential flow filtration (SPTFF) [28] for clarification and the use of periodic countercurrent chromatography (PCC) and simulated moving bed chromatography (SMB) [29] for the capture and purification/polishing steps. This is achieved by coupling the perfusion bioreactor to the ATF filtration, where the flow of liquid alternates across a membrane in a tangential manner, enabling continuous retention of cells while removing spent media or waste in a cyclic process. The clarified material, containing the product, can then be transferred to subsequent capture and polishing steps using PCC and SMB, where the typical chromatographic operations, i.e., load, wash, elution and regeneration, are run in series by multiple columns in a cyclic manner [26, 30]. A fully integrated continuous manufacturing provides advantages by reducing the costs, improving the quality while speeding up the product release [24, 26]

With the biopharmaceutical industry going through a paradigm shift towards continuous manufacturing, continuous monitoring becomes more needed than ever to ensure process control. Integration of PAT into the process stream to measure the CQAs to optimize the process parameters is essential. Implementation of PAT in DSP has been somewhat limited due to various difficulties associated with it [26]. Unlike upstream processing, where several days are needed for cell culture and therapeutic protein production, the purification of the target product in downstream processing takes only a few hours [31]. While response times of up to 1 h are still sufficient for USP, process decisions must be made within seconds for DSP [32], demanding technologies offering faster measurements. Adding to this challenge is the inherent complexity of therapeutic proteins due to their higher molecular weights, multiple post-translational modifications (PTMs), higher order structure variants (HOS) and/or putative molecular interactions [33].

In downstream processing, ensuring the purity of the therapeutic product is of the highest priority. The common CQAs to be monitored include process-related impurities such as host cell proteins (HCP) and nucleic acids, as well as product-related impurities such as higher and lower molecular weight species (HMW, LMW) and charge variants [34]. Different structures of the same protein are often present in biopharmaceutical preparations, making them a heterogeneous mixture, including post-translational modifications such as glycosylation, oxidation, deamidation, formation of aggregates and fragments impacting the stability and efficacy of the drug [35]. N-linked glycosylation is the most common PTM and it impacts the biological activity, pharmacokinetics and immunogenicity of the therapeutic protein [36]. The formation of aggregates during the purification process is an important attribute to monitor, as protein aggregates have shown to exhibit immunogenicity, leading to adverse effects [37]. The inherent complexity of the proteins and the demands of DSP require PAT tools that can deliver faster and high-throughput measurements for ensuring process efficiency and product quality.

The comprehensive monitoring of all critical quality attributes (CQAs) cannot be achieved with a single analytical technology; instead, an integrated approach combining multiple techniques, sensors and control systems is essential for effective process monitoring and control [20, 26]. In continuous downstream processing, characterized by constant fluid flow, PAT plays a pivotal role by enabling the integration of in-line, on-line and at-line technologies. Techniques such as spectroscopy, chromatography and biosensors have emerged as promising PAT solutions. The subsequent sections of this review delve into the latest advancements in PAT tools for downstream processing.

3 | PATs in Protein DSP

PAT facilitates biomanufacturing through diverse analytical techniques and chemometric models [38]. PAT is an umbrella term and includes the components of measurement systems, multivariate tools for predictive modelling and associated control strategies [19]. Measurements can be made real time or near–real time by in-line, on-line and at-line analysis. However, the need of sample pretreatment can be a hurdle in the implementation of analytical techniques in an in-line or on-line fashion. Manual sample acquisition and preparation also introduce variability due to human errors in sample handling [39]. Advances in automated sampling methods have been particularly useful to tackle this challenge. Various robotic liquid handling workstations have been commercialized that can automate sample preparation and pretreatment. Agilent Bravo, Kingfisher Flex, TECAN, Hamilton Microlab, Formulatrix and Beckman Coulter Biomek are some examples of such automated liquid handling platforms [40–42]. There have also been recent attempts to develop custom end-to-end platforms which can perform automated sample preparation and analysis [43, 44]. Automated sampling systems such as MAST and Seg-Flow can draw the samples from the bioprocess stream and deliver them to the analytical devices for ensuing seamless analysis [45–47].

Spectroscopy had been the most common technique employed for real-time analysis due to its non-destructive nature and ease of in-line or on-line integration to the process stream. However, with advancements in analytical instrumentation and automated sampling methods, various techniques such as on-line chromatography and in-line integration of biosensors are being explored for PAT. Emerging PAT techniques for downstream process monitoring are illustrated in Figure 3. For real-time monitoring, analytical measurements are usually followed by chemometric model building, enabling dynamic process control. In subsequent sections, recent advances in analytical techniques (Sections 3.1–3.4) as well as data workflow, including model building and process control (Section 3.5) are discussed (Figure 4).

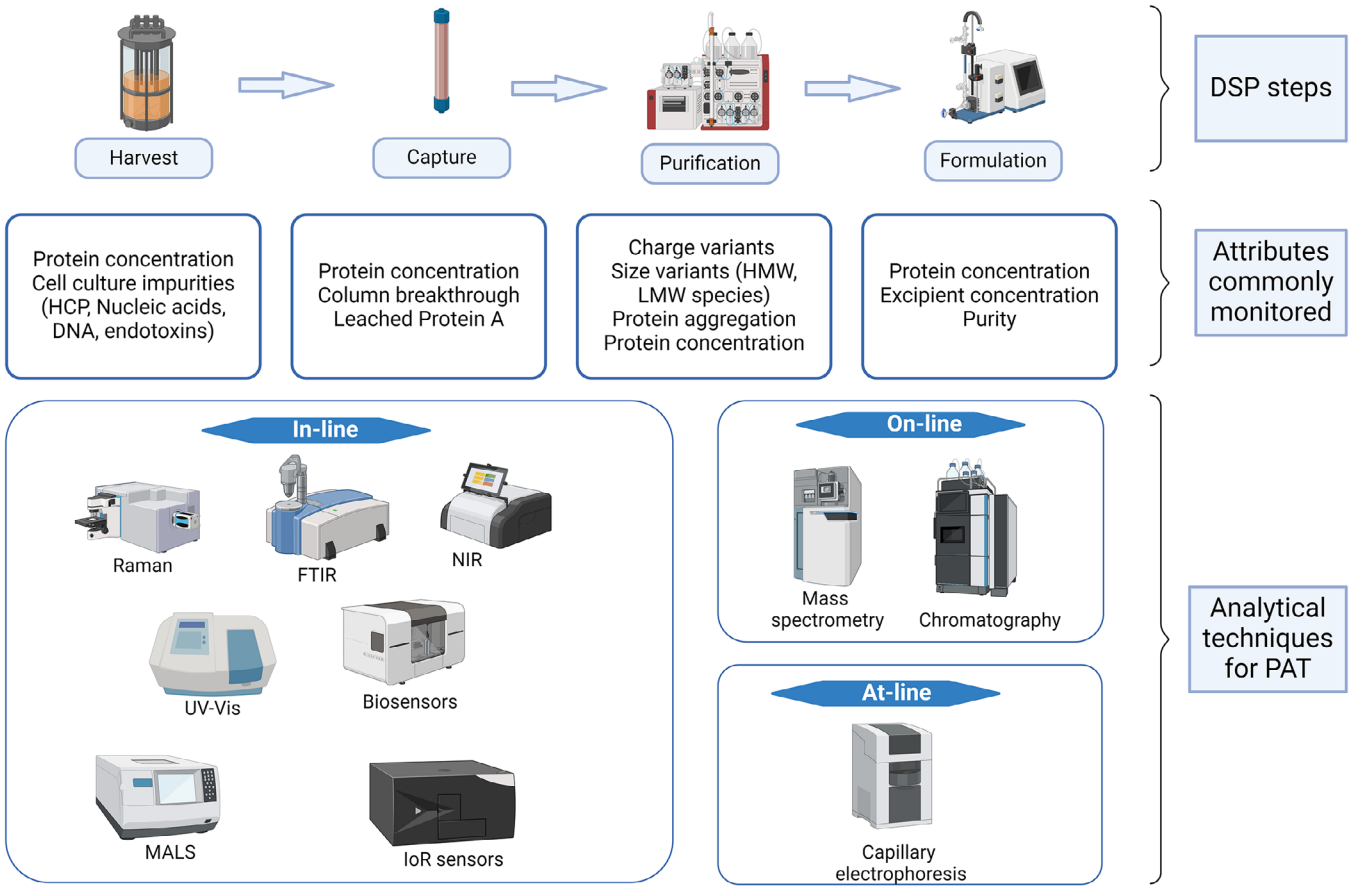

FIGURE 3 | Emerging PAT techniques for downstream process monitoring.

3.1 | Spectroscopic Techniques

Various spectroscopic techniques have been used as PAT tools. Vibrational spectroscopic techniques include Raman spectroscopy, Mid-infrared spectroscopy (MIR) and near-infrared spectroscopy (NIR), which identify molecules based on the vibrations of their bonds. Ultraviolet-visible (UV-Vis) spectroscopy, on the other hand, identifies molecules based on electron energy transitions. Spectroscopic techniques are a method of choice as a PAT tool because (i) they can give quantitative and qualitative information about multiple analytes in a sample and (ii) they can be easily integrated in an in-line setup by the use of a flow cell or a fibre optic probe. In real-time monitoring, spectroscopic measurements are often integrated with multivariate data analytics (MVDA) tools to analyse and extract meaningful information from large spectral datasets [48].

3.1.1 | Infrared Spectroscopy

MIR and NIR are the two most commonly used Infrared spectroscopy techniques in process analytical technology.

3.2 | MIR

MIR spectroscopy (wavelength range from 2500 to 25,000 nm) identifies molecules based on their fundamental vibrations, as they absorb mid-infrared light at specific wavelengths. The absorptions correspond to the changes in the dipole moments of the molecules, offering a 'molecular fingerprint', thereby allowing for a detailed identification of chemical bonds and functional groups [49]. As MIR is based on fundamental vibrations, it can provide direct information on bond-specific vibrations, making it a powerful technique for identifying and quantifying specific molecular components and structural details in biomolecules. MIR spectroscopy applied with the Fourier transform is useful in measuring product concentrations in complex matrices [50].

The high specificity and sensitivity of the MIR come from the highly absorbing nature of biomolecules in this spectral region [51]. The most prominent bands of proteins are found at amide I (~1650 cm^{-1}) and amide II (~1550 cm^{-1}) regions. One limitation of MIR is the strong absorption band overlapping with the amide I region (~~1645 cm^{-1}) for water, usually in the molar range, whereas for protein, the absorption is in the mM to μM range leading to rapid signal saturation, requiring water absorbance subtraction [52]. MIR also requires extensive sample preparations, with the solvents that doesn´t have absorbance in this spectral region to minimize the spectral interference [51].

However, with the attenuated total reflectance (ATR) and Fourier transform infrared spectroscopy (FTIR), these limitations have been mitigated to a larger extent. FTIR enables the measurement of product concentrations in complex matrices [50]. ATR uses an internal reflection element (IRE) for attenuated total internal

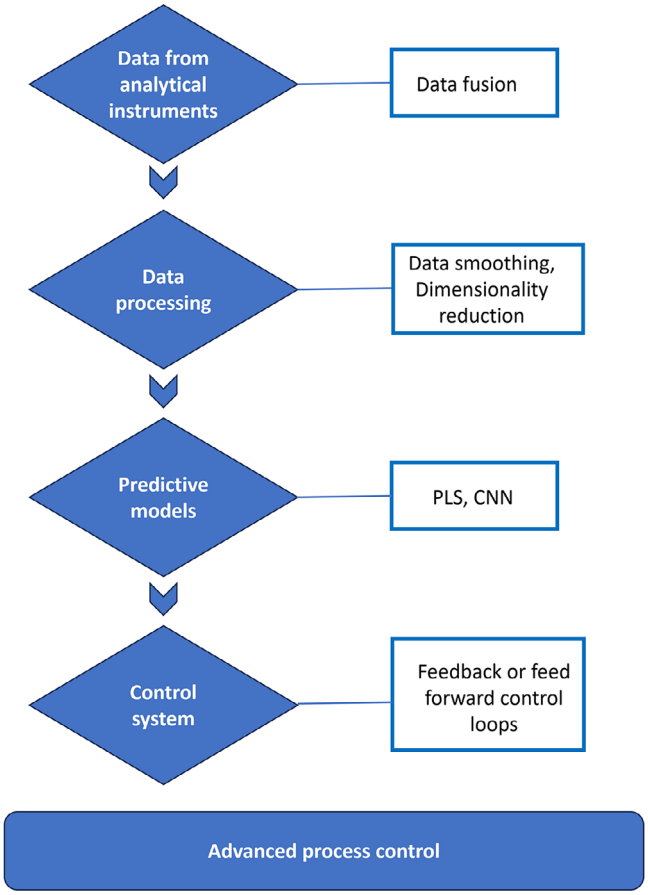

FIGURE 4 | An overview of the data workflow involved in bioprocess monitoring.

reflection, so that when a sample is placed in close contact with the IRE, only a thin layer is probed, preventing absorption saturation by interfering molecules such as water [53].

ATR-FTIR can monitor multiple parameters in real time, and it is an effective tool when combined with algorithms to make real-time prediction and control. It has already been employed for the monitoring of protein downstream process steps in an at-line fashion [54]. The use of ATR fibre optic probes makes it easy to integrate them in-line. Recently, ATR-FTIR has also been implemented in-line during the UFDF step, to monitor the protein concentration with a simple one-point calibration algorithm [55] or both protein and various excipient concentrations, with a multi-variate data analysis (MVDA) model [56]. In both cases, rapid measurements in seconds and accurate concentration predictions were achieved. Advances in MIR such as surface-enhanced infrared absorption spectroscopy (SEIRA) and tip-enhanced IR methods [57, 58] can increase the measurement sensitivity by several folds and have already been explored as in situ monitoring tools [59], showing great potential to also be considered as a PAT tool for downstream processing.

3.3 | NIR

NIR spectroscopy (wavelength range 800–2500 nm), induces overtone and combination vibrations rather than fundamental vibrations, as seen in MIR. These higher energy vibrations appear as broad and often overlapping peaks in the NIR spectrum, providing a more generalized but rapid assessment of molecular features. NIR is particularly sensitive to bonds involving hydrogen, such as C—H, N—H and O—H bonds, making it well-suited for probing water content and organic molecules in complex matrices [60].

The absorbance of molecules in the NIR region is several times lower than MIR, enabling deeper penetration and requiring minimal sample preparation [51]. While less specific than MIR due to broader peaks, NIR's ability to penetrate deeper into samples and accommodate high-throughput analysis [60] makes it invaluable in monitoring concentrations and detecting variations in chemical composition, especially when combined with chemometric models for quantitative analysis. The FDA stresses the importance of building reliable chemometric models for NIR measurements [61]. NIR's ability to be used directly on samples without the need for extensive sample preparation [51] makes it highly applicable for real-time analysis, where rapid measurements are needed.

NIRs have been employed in an on-line setup to monitor the PEGylation of proteins, which is an important quality attribute to monitor during the downstream processing of conjugated proteins. NIRS spectra were collected in real time for every 3 s, which was then followed by model building for information extraction and process control [62].

Similar to MIR, Fourier transform techniques can also be used with NIR. Recent studies have used FT-NIR for downstream process monitoring. Thakur et al. monitored the column loading using an on-line FT-NIR setup, where the target product concentration in both the harvested broth and column effluent from Protein A chromatography was measured in real time, for every 3 s and column breakthrough was detected successfully with high accuracy [63]. It has been integrated in-line for the simultaneous monitoring of protein and excipient concentrations in antibody formulations during the UFDF, and the quantification was done in real time, in a few seconds [64]. In both cases, real-time process control was achieved.

FT-NIR has also been utilized for monitoring continuous processing, specifically for measuring the concentrations in feed and retentate streams during the UFDF step with single-pass tangential flow filtration (SPTFF). By applying appropriate calibration models, accurate concentration predictions can be achieved, facilitating effective process control [65].

3.3.1 | Raman Spectroscopy

Raman spectroscopy is based on the inelastic scattering of photons, where a small fraction of the incident light undergoes inelastic scattering, resulting in energy shifts corresponding to the vibrational modes of the specific molecules present. These shifts, called Raman shifts, allow the identification of the molecular composition and structural information of the sample. While IR is sensitive to dipole changes, the Raman effect is sensitive to polarizability changes [66]. Therefore, IR and Raman can be

used to get complementary information, useful for characterizing chemical and structural attributes.

Raman requires minimal sample preparation and is non-invasive and non-destructive to samples. It is one of the most widely used techniques in PAT, and with the advent of machine learning and model development, accurate detection and predictions can be done. The development of portable flow cells for Raman [67] has made this technique easy to implement inline or online to monitor quality attributes. Multiple studies have been published in recent years where Raman was employed for real-time monitoring of various downstream processing steps.

Chen et al. employed Raman spectroscopy for in-line CQA monitoring of IgG1 monoclonal antibody (mAb) during the Protein A chromatography process, where they developed a predictive model showing high predictive accuracy for various CQAs including target protein concentration and aggregates [38]. In Nitika et al. [68], Raman spectroscopy coupled with a convolutional neural network framework allowed for on-line real-time monitoring and determination of charge variants during cation exchange chromatography (CEX) of mAbs. In Vasko et al. [69], an in-line monitoring of process impurities during recombinant protein purification was developed using calibration models based on Raman and NIR spectroscopy. Wang et al. implemented Raman spectroscopy in-line for measuring product aggregation and fragmentation in real time, developed a calibration model, achieving accurate quality measurements every 38 s. These authors integrated hardware automation and machine learning techniques to increase data throughput [70].

Raman spectroscopy has also been utilized during the harvest step to measure mAb concentrations in real time from the permeate stream of a perfusion bioreactor. By integrating Raman with chemometric modelling, accurate concentration predictions can be achieved. This demonstrates the effectiveness of Raman spectroscopy as a PAT tool in continuous manufacturing settings [71].

The advantage of Raman spectroscopy over IR spectroscopy is that Raman spectra have minimal water interference and thus can be used in analysing aqueous samples or biological specimens where water is a significant component without compromising spectral clarity. One of Raman's limitations is its sensitivity to fluorescent backgrounds, which can obscure Raman signals in certain samples [72]. Raman is also sensitive to the sample temperature, with higher temperature causing diffuse Raman lines, requiring temperature adjustments. Raman signals are often weaker than those in other spectroscopic methods, leading to challenges in detecting low-concentration analytes [73].

Many of these limitations have been addressed through recent advancements, with the development of multiple variants of Raman spectroscopy techniques offering unique advantages over traditional methods. There have been efforts to reduce the fluorescence interference in Raman signals by using red or deep-red laser wavelengths, time-resolved Raman and shifted excitation Raman difference spectroscopy (SERDS). Enhanced variants of Raman include resonance Raman, surface-enhanced Raman (SERS), surface-enhanced resonance Raman (SERRS) and tip-enhanced Raman (TERS), all of which are used for their inherent advantages including increased Raman signal, reduced limit of detection and the usage of reduced sample volume [74]. For a more detailed discussion on surface-enhanced Raman techniques, and their implementation, readers are encouraged to refer to the excellent review articles available on this topic [74, 75]. Advanced Raman spectroscopic techniques depend on expensive instrumentation, often hindering their routine implementation. However, costs are anticipated to decrease over time, enabling broader adoption within the biopharmaceutical industry [73].

In an attempt to reduce operating costs, Goldrick et al. explored the potential of a high-throughput Raman spectroscopy microscope in monitoring different elution conditions and monomer purity in a cation exchange chromatography (CEX) step of a Fc-fusion protein in downstream processing. Combined with chemometric modelling, this method was able to classify samples based on protein concentration and monomer purity. However, this method was not able to accurately detect low and high molecular weight species. However, the classification of samples based on concentration and purity allowed the prioritization and reduction in samples analysed by the regular HPLC and UV setup [76]. The authors claim that this method has the potential to reduce the capital and operating costs by reducing the reliance on multiple separate analytical techniques. Further adaptations and studies are required to demonstrate the effectiveness of this technique for downstream process monitoring.

3.3.2 | UV-Vis Spectroscopy

UV-Vis Spectroscopy (wavelength range 10–800 nm) is based on electron transition rather than vibrational states as seen in Raman or IR. The absorption of ultraviolet (10–400 nm) and visible (400–800 nm) light by a sample causes the electrons to transition from the ground to the excited state. These transitions are characteristic of bonds and functional groups, and the absorbance of light is directly proportional to the analyte concentration. The resulting absorbance spectrum therefore provides quantitative information about the concentration and chemical structure of the analyte [77].

Since UV-Vis primarily detects chromophores, molecular regions where electron excitation is more likely, it is particularly useful for analysing conjugated systems, including proteins and nucleic acids, and monitoring concentration changes in real time [77].

Rolinger et al. employed both Raman and UV in an in-line setup to measure the mAb concentration in the column effluent during the load phase in Protein A chromatography. This was combined with heavy data processing to derive insights from the spectral data, including multiple steps of data pre-processing, modelling using PLS and CNNs followed by the fusion of both datasets. In the end, the study concluded that, UV-data-based PLS modelling alone had superior performance in predicting the mAb concentrations [78].

Another study employed a dual wavelength LED photometric sensor configured at two different wavelengths, where UV absorbance was used in determining the protein concentration during the tangential flow filtration process (TFF) [79].

A notable advancement in UV-Vis analytical methods is the development of variable pathlength slope (VPE) instruments, which employs multiple pathlengths to measure the absorbance automatically at a specific wavelength [80]. Accurate measurements of protein concentrations can be made with VPE, even with high-concentration proteins without requiring dilutions [81]. VPE in conjunction with a flow cell can be easily integrated in-line for various unit operations, enabling real-time acquisition of UV signals [80]. Various instruments using the variable pathlength technology have been commercialized under brand names CTech SoloVPE, CTech FlowVPE and CTech FlowVPX. A variable pathlength UV-Vis spectrometer has already been utilized for the in-line monitoring of protein concentration during the UFDF on a lab scale [82]. However, there are some challenges in implementing VPE systems as they are computer-controlled, can consume a significant footprint, involve various interchangeable components and require routine cleaning and clearance verifications [79].

3.4 | Chromatography and Hyphenated Techniques

Chromatographic separation of compounds based on size exclusion or ion exchange has been regularly employed in product characterization in routine testing. Chromatography coupled with a suitable detector can efficiently identify CQAs such as charge variants, size variants and glycosylation patterns [83]. This had not been previously considered for real-time analysis due to the time taken for separation of compounds by chromatography.

Advances in automated sampling solutions have made it possible to employ chromatography in an on-line setup, where the sample solution can be automatically drawn from the process stream and passed on to the chromatographic analysis. An on-line ultra-performance liquid chromatography (UPLC), based on ion exchange, had been employed by Godbole et al., in 2024 to determine the ratio of co-formulated mAbs. The UPLC was able to effectively distinguish and identify the co-formulated antibodies despite their high structural similarity [84] which would have been challenging with other techniques such as spectroscopy. On-line UPLC therefore enabled in-situ, automated real-time physical separation of the antibodies and their detection. Though this significantly reduced the analytical turnaround time, it still took 30 min of measurement time per sample [84], highlighting the need for speeding up the separation time to be able to use chromatography as a realistic PAT tool.

Despite the limitations, adaptations of the chromatography setup for real-time analytics continue to evolve. A specialized HPLC setup, designed especially for real-time monitoring, with automated sampling and data processing has been developed for monitoring various CQAs such as size variants and maximum column loading capacity, during capture and purification steps in downstream processing. This setup achieved faster measurements with shorter sampling times ranging from 1.30 to 2.35 min [85].

Mass spectrometry has been extensively used in off-line analysis of drug products due to its high throughput and sensitivity, usually hyphenated with chromatography. It can detect a wide range of quality attributes, such as glycosylation patterns [86], charge variants, aggregates and process-related impurities like host cell proteins (HCP) [87]. However, it has not been considered for PAT as it is not feasible to implement in-line or on-line, due to its need for sample preparation and associated complexity [88].

A solution to this limitation was explored by Diehm et al., in 2024, by hyphenating a micro-simulated moving bed chromatography (μSMB) for sample preparation and using MS for measurements. This was implemented in an on-line setup within the PAT. Unlike traditional chromatography methods, where there is more time needed for separation, μSMB was able to process the sample in a significantly lower amount of time (2–8 min), leading to continuous MS-measurements of CQAs [88].

A significant advancement in the field of mass spectrometry is the development of multi-attribute methods (MAM) for monitoring multiple quality attributes of a biotherapeutic simultaneously. It is a targeted and optimized analytical solution that uses a combination of high-resolution MS data and suitable software enabling automated identification and relative quantification of important PTMs [89]. MAM has become a valuable tool for the quality control of biotherapeutics [90]. Its utility for real-time analysis in a PAT setting was recently explored where an end-to-end automated online MAM system was developed for quality attributes monitoring during upstream manufacturing [91]. The demonstrated capabilities of MAM make it a promising tool to be also explored for DSP workflows. For a more detailed discussion on MAM applications in biopharmaceutical analysis, readers are encouraged to refer to the comprehensive review in ref. [92].

Overall, chromatography coupled with mass spectrometry or other detection techniques has been extensively utilized for off-line analysis, offering high specificity, sensitivity and the ability to simultaneously monitor multiple attributes. However, its integration into real-time environments presents substantial challenges. Advances such as micro-SMB chromatography have significantly decreased sampling and measurement times, yet these remain on the order of minutes, which is considerably slower than the seconds-scale response achievable with spectroscopic methods. While on-line implementation of chromatography is technically feasible, in-line integration has so far been inherently impractical due to the operational nature of the technique. These constraints underscore the limitations of chromatography for real-time process control in downstream processing, where rapid and dynamic monitoring is essential.

3.5 | Biosensors

Various biosensing techniques have been employed as PAT tools in an attempt to improve the specificity, sensitivity, speed and ease of detection. Fluorescence-based biosensors use fluorescently labelled ligands to bind to the target analyte, and the detection is based on the change in fluorescence intensity. Immobilized fluorescent reporters had been recently employed in the monitoring of the capture step in downstream processing, where column breakthrough of 5% could be detected [93]. Miniaturization of analytical techniques enables shorter operation times, requires only a small sample volume and can be implemented

easily in-line or on-line. Pedro et al., explored this potential by miniaturizing a fluorescence dye–based microfluidic sensor, and the measurement of protein aggregation was performed in under 10 min. This was however done in an at-line setup and its performance in an in-line or on-line setup is yet to be investigated [94].

Localized surface plasmon resonance (LSPR)-based biosensor has recently emerged as a promising technique for real-time monitoring. LSPR arises from the collective oscillations of free electrons in noble metal nanoparticles, such as gold or silver, when irradiated with visible light, producing a distinct extinction band known as the LSPR band. LSPR biosensing detects biomolecular interactions by monitoring shifts in this band. When a target protein binds to a ligand immobilized on a sensor surface containing these nanoparticles, it alters the local refractive index near the nanostructures, changing the resonance conditions. This optical shift, indicative of the binding event, is recorded in real time with picometer (pm) precision [95, 96].

LSPR biosensors have been utilized for in-line monitoring of the Protein A capture step, enabling the high-sensitivity detection of column breakthrough by measuring product concentrations as low as 2 μg/mL in the effluent, allowing for early identification of column leakage [96]. Binding properties can also be exploited to measure different attributes, other than concentration measurements. For example, aggregates have enhanced apparent affinity due to their interactions with multiple ligands. Based on this principle, a distinction between aggregates and monomers can be made [97]. This avidity effect leading to higher binding response in the LSPR biosensor has been utilized to monitor protein aggregates during affinity purification, enabling the in-line detection of aggregates as low as 1% [98].

LSPR biosensors are less sensitive to changes in temperature, sample composition and vibrations, and unlike spectroscopic techniques, they do not require advanced data analysis to extract product concentrations [96]. It also has a short response time where the measurements can be done in a few seconds and a large, tunable dynamic range with little need for sample pretreatment [99].

However, a key challenge lies in the dependence on highly specific ligands, as the sensitivity and specificity of LSPR biosensors are dictated by ligand selection. Identifying suitable ligands can be difficult for certain analytes. Despite this, the technique shows great promise for real-time process monitoring due to its straightforward integration for in-line applications, compact setup and rapid measurement capabilities.

Various biosensing techniques are constantly emerging, examples include whispering gallery mode (WGM) resonators where Single binding events in small volumes can be measured because of its robust optomechanical features [100] and biosensing by particle motion (BPM), an affinity-based sensing technology with single particle and single molecule resolution [101]. These techniques have already been explored for biomarker detection applications [102, 103]. The inherent advantages of these techniques enabling label-free detection with high sensitivity make them promising tools for continuous process monitoring.

3.6 | Other Emerging Techniques

Other than the most common types of techniques discussed above, various other techniques have been explored as a PAT tool for real-time protein analysis. Multi-angle light scattering (MALS), capillary electrophoresis and index-of-refraction-based techniques are some of the recently explored ones.

MALS is a light scattering–based technique that measures the intensity of scattered light at different scattering angles and correlates it to the molecular weight and concentrations of proteins [104]. MALS has been regularly used for protein characterization [105]. Its application in real-time protein analytics was explored by using it in an in-line setup during the mAb purification to monitor the protein aggregation, which led to real-time results and immediate feedback control [106]. However, this has been the only study so far to explore MALS as a PAT tool in downstream processing, and further studies are needed to fully understand the capability of this technique.

Capillary electrophoresis, a separation technique based on the differential migration of charged species in an electric field, had been widely used in the biopharmaceutical industry for analytical characterization. However, longer measurement times had been a bottleneck for the technique to be actually considered for real-time analysis [107]. Capillary electrophoresis coupled with a UV detector was implemented to monitor the mAb isoforms in the eluents of CEX columns. Though this was only performed in a setup similar to at-line analysis, the method utilized sequential injections, greatly reducing the time taken for analysis. With this modified approach, this might be a potential analytical technique that is worth exploring for on-line PAT setup in future research [108].

Harris et al. employed a sensor based on Index of Refraction (IoR) forming an in-line setup to measure the protein concentrations in real time during the capture and ultrafiltration steps of downstream processing. The sensor was able to accurately measure the protein concentrations during ultrafiltration and during the capture step [109]. One challenge of IoR is that, the signal can be impacted by the physiochemical properties of the fluid being measured, and further studies are needed to ensure the effectiveness of this technique as a PAT tool.

3.7 | Chemometrics and Process Control

In continuous manufacturing, real-time monitoring of quality attributes is essential to maintain process control and ensure product consistency. Achieving reliable real-time analysis requires not only high-precision measurements but also accurate interpretation of complex data. Recent advances in analytical technologies have significantly improved both the selectivity and sensitivity of various techniques, yet leveraging this potential relies on precise data interpretation for predicting the CPPs and CQAs, which are essential for real-time process control and quality assurance. This has led to a growing emphasis on statistical modelling to extract meaningful insights from the extensive datasets generated. With the integration of machine learning, there is tremendous potential to further optimize data utilization, enabling the development of predictive models that

can accurately forecast quality outcomes or key process parameters. This shift towards data-driven, predictive analytics marks a paradigm shift in real-time monitoring, making continuous analysis a feasible and transformative reality in manufacturing. The field of chemometrics, which merges statistical and chemical approaches, encompasses these efforts for data interpretation and predictive analysis. Essentially, chemometrics involves applying multivariate, empirical modelling techniques to chemical data [110].

The raw data, especially from the spectroscopic measurements needs extensive data pre-processing such as baseline correction and spectral smoothing [111]. This is followed by the application of advanced statistical techniques. Multivariate data analysis plays a crucial role in extracting meaningful insights from the complex datasets generated in process monitoring. Techniques such as Principal Component Analysis (PCA) are applied for dimensionality reduction, which facilitates the interpretation of high-dimensional data [110].

There is a growing emphasis on data-driven models to predict quality outcomes, enabling process control. The basic principle of these models is that they use a mathematical function linking a set of predictors (independent variables) to the responses (dependent variable) learned directly from the historical data [112]. Data-driven models are particularly useful because, they rely on historical data for predictive analysis, and it is highly applicable where process parameters and product quality are not clearly understood [112].

There are multiple data-driven models used in predictive modelling, and the common ones include random forest regression (RFR), partial least square regression (PLSR), Gaussian process regression (GPR), and more recently, the use of artificial neural networks (ANNs). Convolutional neural networks (CNNs) are a special subtype of ANNs that have gained popularity and have been used in the field of chemometrics [112]. Often referred to as 'soft sensors', these models are used to predict the CQAs and CPPs based on the correlation with generated data. They form the basis for real-time monitoring and can be integrated within a controller to enhance process control [113]. Almost all of the recent studies employing PAT tools for downstream process monitoring (Table 1) have extensively used chemometrics, employing one or more of the above-mentioned models to predict the quality outcomes, thereby enabling real-time process control.

Recently, there has been more focus on constructing digital twins (DT), a virtual and digital representation of physical objects or the process. DTs can be constructed for each unit operation, modelling the process in a virtual fashion, which enables real-time predictions and process control. For instance, DTs have been recently constructed for ion-exchange chromatography (IEX) processes, and are systematically integrated with the HPLC system and a server computer. The DT was able to predict the elution profiles, enabling automated collection control [114].

There has been more and more emphasis on the integration of cyber-physical systems, where a range of analytical techniques will be connected to automated data processing workflows, leading to efficient data management, visualization and utilization of augmented reality to enable dynamic process control. Helgers et al. demonstrated the integration of various spectroscopic techniques, including Raman, FTIR, fluorescence spectroscopy and UV-Vis, with a predictive modelling data workflow and DT to achieve advanced process control (APC) [115].

Overall, the integration of various in-line and on-line PAT techniques to the bioprocess stream, and a process monitoring system (PMS) at the analytical and bioprocess interface enabling automated data analysis and visualization, enables feedback or feedforward control of the analytical and process instruments, ultimately resulting in dynamic process control and thereby making real-time release testing (RTRT) possible [40].

4 | Summary and Outlook

Here we reviewed the emerging analytical techniques for implementation within PAT and provided a perspective of recent advancements in data workflow and automation which are key drivers in realizing QbD and RTRT. Although significant progress has been made in analytical technologies to enhance throughput, sensitivity and measurement speed, each technique has its own unique advantages and limitations.

Spectroscopic techniques have been widely employed in PAT, due to their non-destructive nature, minimal sample preparation requirement and ease of in-line or on-line implementation. However, they still fall behind in terms of offering high specificity, requiring heavy and advanced data processing to derive meaningful insights. In contrast, chromatography, and its hyphenated techniques such as mass spectrometry offer the required specificity. Challenges related to extensive sample preparation and pre-processing have been addressed through automated sampling solutions. Furthermore, advances in chromatographic instrumentation have significantly reduced measurement times. However, the measurement times are still longer for true real-time analytics in DSP, where response time within a few seconds to minutes is essential. Additionally, on-line implementation poses challenges, and in-line implementation currently remains largely impractical.

Biosensors can fill in the much-needed gap, by offering high specificity, while being easy to implement in-line or on-line, delivering faster measurements required for real-time control. When equipped with suitable ligands, they provide the necessary specificity to detect not only the target analyte, but also other product- or process-related impurities, thereby making them a platform tool for monitoring multiple attributes. However, biosensors require ligands with high affinity for the target analyte, which can sometimes pose a bottleneck. Though biosensing-based techniques look promising, studies demonstrating their application in real-time monitoring are limited, indicating a clear need for further research.

Advancements are continually evolving at the analytical front, such as surface enhancement for spectroscopic methods [58, 75] and miniaturization of chromatography and mass spectrometry [116, 117]. These developments offer unprecedented sensitivity and facilitate easier in-line and on-line integration. Even techniques once considered impractical for PAT implementation,

TABLE 1 | Summary of recent literature (past 5 years) on employing various analytical technologies for real-time analysis of protein downstream processing.

Technique	Mode of PAT implementation	Downstream processing step	CQAs monitored	Reference
Raman	In-line	Purification	Target protein concentration, aggregates	Chen et al. [38]
Raman	On-line	Purification	Charge variants	Nitika et al. [68]
Raman	In-line	Purification	Process impurities	Vasko et al. [69]
Raman	In-line	Purification	Product aggregation and fragmentation	Wang et al. [70]
Raman	In-line	Harvest	Protein concentration	Yilmaz et al. [71]
MIR (FTIR)	In-line	UFDF	Protein concentration	Milewska et al. [55]
MIR (FTIR)	In-line	UFDF	Excipients, protein concentration	Wasalathanthri et al. [56]
NIR	On-line	Capture	Protein concentration, Column breakthrough	Thakur et al. [63]
NIR	In-line	UFDF	Protein and excipient concentration	Thakur et al. [64]
NIR	On-line	PEGylation	MonoPEGylated and multiPEGylated proteins	Hebbi et al. [62]
NIR	In-line	SPTFF (UFDF)	Protein concentration	Thakur et al. [65]
Raman and UV	In-line	Capture	Protein concentration	Rolinger et al. [78]
UV dual wavelength	In-line	UFDF	Protein concentration	Westwood et al. [79]
μSMB-MS	On-line	Purification	Protein concentration	Diehm et al. [88]
UHPLC	On-line	Capture, Purification	Maximum column loading capacity, size variants (LMW, HMW species)	Graf et al. [85]
UPLC	On-line		Total protein concentration, Ratio of co-formulated mAbs	Godbole et al. [84]
Fluorescence-based detection	On-line	Capture	Protein concentration, Column breakthrough	Goyal et al. [93]
Fluorescence dye–based microfluidic sensor	At-line	Viral inactivation, Purification	Protein aggregation	Pedro et al. [94]
LSPR	In-line	Purification	Protein aggregation	Tran et al. [98]
LSPR	In-line	Capture	Protein concentration, Column breakthrough	Tran et al. [96]
Capillary electrophoresis	At-line	Purification	Charge variants (mAb isoforms)	Kumar et al. [108]
Index of refraction	In-line	Capture, Ultrafiltration	Protein concentration	Harris et al. [109]

such as chromatography and mass spectrometry have become viable PAT tools because of these innovations.

Each analytical technique within the PAT framework offers distinct advantages and limitations. However, the strength of PAT lies in its flexibility to employ the most suitable techniques for monitoring specific quality attributes based on process requirements. The emphasis should extend beyond individual techniques to optimizing the integration and effective utilization of acquired data. Predictive modelling plays a pivotal role in deriving actionable insights for effective process control. Enhancements in data automation, streamlined workflows, the establishment of data pipelines connecting enterprise systems and predictive modelling, combined with DT, hold the potential to realize the vision of real-time process monitoring and real-time product release.

To fully implement Quality by Design within the framework of continuous manufacturing, a comprehensive PAT workflow must go beyond assay automation and high-throughput analytics. Equal emphasis must be placed on developing robust multi-layer cyber-physical systems with effective risk mitigation strategies to enable efficient data management, real-time visualization, augmented reality and Internet of Things (IoT) infrastructure. Together, these advancements will facilitate real-time analytics and process control, bringing the biomanufacturing industry closer to realizing fully integrated, intelligent manufacturing processes.

Although it is widely acknowledged that a PAT framework aligned with a QbD approach is necessary to achieve continuous manufacturing, process control and RTR) [40], there is still some hesitancy to implement PAT on a commercial scale. This reluctancy is largely due to the perceived increased burden of regulatory submissions. With the growing reliance on predictive models for process control, it is essential to validate these models and provide rigorous documentation. This includes demonstrating their comparability, reliability and justification for the parameters used during model development. Additionally, the comparability of routine analytical data with PAT-generated data should be established [118].

Nevertheless, the FDA has been actively encouraging industries to adopt PAT and transition to a QbD approach. Since the introduction of PAT in 2004 [18], significant efforts have been made to support industries in this transition. To further support this initiative, the FDA established the 'Emerging Technology Team' (ETT), which collaborates with the pharmaceutical industry to provide early assessments and guidance during regulatory submissions for PAT, ensuring a smoother review process [119].

The biopharmaceutical industry is experiencing a paradigm shift in manufacturing, analytics and quality control. While significant emphasis is placed on advancing analytical instrumentation and optimizing data workflows, it is equally critical to focus on implementing these advancements at a commercial scale. Open collaboration between regulatory authorities and industry professionals is essential to realize the vision of effective QbD implementation, fostering innovation while ensuring compliance and product quality.

Author Contributions

Pavithra Sathiyapriyan: conceptualization, writing–original draft. **Shatanik Mukherjee**: conceptualization, supervision. **Thomas Vogel**: writing–review & editing. **Lars-Oliver Essen**: supervision, writing–review & editing. **David Boerema**: resources. **Martin Vey**: resources. **Uwe Kalina**: resources, supervision.

Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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