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REVIEW

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Opportunities and challenges of real-time release testing in biopharmaceutical manufacturing

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Abstract

Real-time release testing (RTRT) is defined as "the ability to evaluate and ensure the quality of in-process and/or final drug product based on process data, which typically includes a valid combination of measured material attributes and process controls" (ICH Q8[R2]). This article discusses sensors (process analytical technology, PAT) and control strategies that enable RTRT for the spectrum of critical quality attributes (CQAs) in biopharmaceutical manufacturing. Case studies from the small-molecule and biologic pharmaceutical industry are described to demonstrate how RTRT can be facilitated by integrated manufacturing and multivariable control strategies to ensure the quality of products. RTRT can enable increased assurance of product safety, efficacy, and quality—with improved productivity including faster release and potentially decreased costs—all of which improve the value to patients. To implement a complete RTRT solution, biologic drug manufacturers need to consider the special attributes of their industry, particularly sterility and the measurement of viral and microbial contamination. Continued advances in on-line and in-line sensor technologies are key for the biopharmaceutical manufacturing industry to achieve the potential of RTRT.

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KEYWORDS

biopharmaceuticals, biopharmaceutical manufacturing, critical quality attributes, process control, real-time release, real-time release testing

1 | INTRODUCTION TO RTRT AND REGULATORY POLICY

The manufacturing of biopharmaceuticals is tightly regulated to assure patients and healthcare providers of the safety, efficacy, and quality of drug products. At a minimum, the process development includes four steps (FDA, 2004; ICH, 2009a):

1. Define the quality target product profile (QTPP),
2. Identify the critical quality attributes (CQAs),
3. Select an appropriate manufacturing and control strategy using risk assessment, and
4. Implement a control strategy.

Given that many regulatory documents and journal publications have described the methodologies for the individual steps in great detail (EMA, 2012; ICH, 2005, 2009a, 2009b, 2010), that information will not be recapitulated here. Instead, the focus of this article is on *real-time release testing (RTRT)*, which is the ability to evaluate and ensure the safety, efficacy, and quality of a final drug

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substance and/or product *based on in-process data with reduced end-product testing* (EMA, 2012; ICH, 2005, 2009a, 2009b, 2010). RTRT is an element of an overall control strategy facilitated by the use of fully automated and integrated manufacturing and testing.[1] The goals of an effective RTRT program are to leverage enhanced process understanding to allow for corrective actions in real time that will increase assurance of quality, shorten process cycle times, lower inventory requirements, reduce end-product testing, and lower overall manufacturing costs (EMA, 2012; ICH, 2005, 2009a, 2009b, 2010; Moore, 2011). The increased assurance of product quality is enabled by increased usage of real-time measurements and their use in control systems.

Regulatory guidance on RTRT is available in ICH Q8 (R2) on pharmaceutical development (ICH, 2009a), and in the U.S. Food and Drug Administration (FDA, 2004) and European Medicines Agency guidelines (EMA, 2012). This article describes technical strategies for and challenges with the application of RTRT in biopharmaceutical manufacturing.

Health authority understanding and agreement of the application of these concepts to RTRT for biopharmaceuticals have been increased by the European Union Guideline on RTRT, the FDA pilot programs on Quality by Design (QbD), and principles of ICH Q8(R2), Q9, Q10, and Q11 (ICH, 2005, 2009a, 2009b, 2012).

1.1 | Strategies for obtaining RTRT for each CQA

The design of an RTRT strategy should take into account the operation of the individual unit operations (e.g., bioreactor, chromatography columns) as well as the operation of the overall plant. A systematic approach for the design of the overall RTRT strategy is (Lu et al., 2015):

1. Build a mechanistic, empirical, or semi-empirical dynamic model for each unit operation (UO) during its development, based on appropriately qualified analytical procedures,
2. Validate each UO model including descriptions of disturbances and model uncertainties and place into a plant-wide simulation,
3. Use each UO model to design a control system to satisfy local operating constraints and meet "local" material attributes,
4. Evaluate performance in simulations and propose design modifications as needed,
5. Implement and verify the control system for each UO, and
6. Use plant-wide simulation to evaluate UO interactions and design and verify a plant-wide control system and RTRT strategy to ensure that the CQAs are consistently met.

Figure 1 is a graphical depiction of this approach.

The construction of any model of a unit operation requires the collection of experimental data during process development. Empirical models such as partial least squares and response surface methods are the most commonly applied in the biopharmaceutical industry due in part to the very high degree of complexity of some of the unit operations and partly to insufficient mechanistic understanding being available to be able to construct a mechanistic model for some of the unit operations. Although empirical models do not provide information on unmeasured states such as compositions within cells, they have the advantage of not requiring deep knowledge to develop, and can provide accurate predictions when the experimental data used to

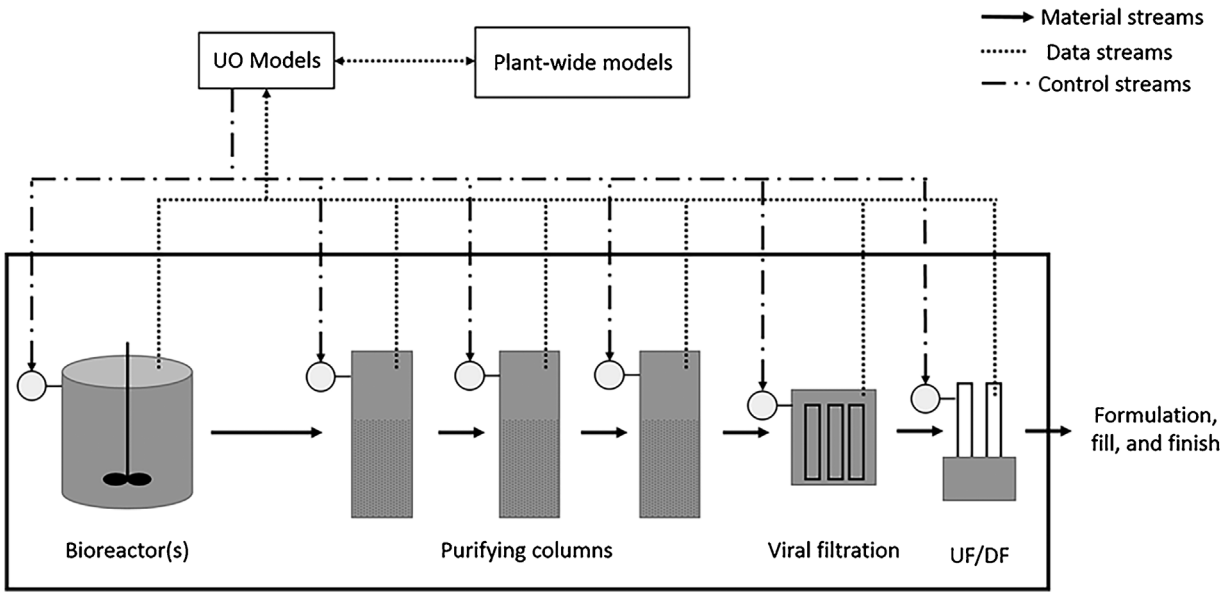

FIGURE 1 The relationship between the manufacturing operation and models for RTRT. Data streams are continually used to make predictions and inform control systems. The unit operations (such as bioreactors or UF/DF) and associated local control systems are designed to satisfy local operating constraints and material attributes, irrespective of whether the operations are at laboratory or production scale. The unit operations and their interconnections are incorporated into a plant-wide model that is used for plant-wide control system design (Jiang & Braatz, 2016; Lu et al., 2015). The successful implementation enables RTRT by ensuring product quality specifications are achieved. Continuous maintenance of these models is performed via comparisons of predictions versus actual

construct the model span the full region of operational space (Jiang & Braatz, 2016; Lu et al., 2015).

Mechanistic models aim to explain and describe unit operations by employing fundamental relations such as mass conservation, thermodynamics, and chemical and biological kinetics (Lu et al., 2015). Mechanistic models require a significant amount of understanding to develop, but their cost of construction can be reduced through reusing models implemented in previous process development (Lu et al., 2015). By using fundamental relations as additional knowledge, mechanistic models require less experimental data than empirical models to achieve the same level of predictive capability. Bioreactor models have been developed that include cell and all molecular species conservation equations in solution, the fluid dynamics of both solution and gas bubble phases, mass transfer between liquid and gas phases (Bezzo, Macchietto, & Pantelides, 2003; Zhang, Zhang, & Shengdi, 2009), and metabolite fluxes between the cells and liquid (Jahic, Rotticci-Mulder, Martinelle, Kult, & Enfors, 2002; Nolan & Lee, 2011), but mechanistic models are not yet well-developed for describing post-translational modifications. Full mechanistic models are well developed for the other unit operations common in biopharmaceutical manufacturing, such as ultrafiltration/diafiltration (UF/DF) (Gefroh & Lutz, 2014; Ho & Zydney, 2000; Polyakov & Zydney, 2013). Full mechanistic models have been developed for some other unit operations, such as chromatography (Borg et al., 2014; Brooks & Cramer, 1992; Karkov, Sejergaard, & Cramer, 2013), but too many molecular species are present in the feed to chromatography columns to be able to fit all of the adsorption and desorption parameters needed in a full mechanistic model. For example, the number of host cell proteins alone ranges in the tens of thousands in bioreactors using mammalian cell lines. To address the high complexity, the most detailed mechanistic models applied in the biopharmaceutical industry for such unit operations collect all of the species other than the target molecule into one group and define lumped parameters for the group, simplify the kinetics, or simplify the isotherms (Karkov et al., 2013). Although simplified mechanistic models require some strong assumptions and/or approximations, such models have demonstrated some predictive capability (Borg et al., 2014; Brooks & Cramer, 1992; Karkov et al., 2013). Even when simplified, mechanistic models have better extrapolation than empirical models, and are more likely to produce accurate predictions when used for process synthesis, optimization, and scale-up.

When needed, some empirical modeling may be incorporated into the mechanistic models. For example, the kinetics of some post-translational modifications are not currently understood well enough for mechanistic models to be constructed with confidence, and empirical models may be needed for predicting variations in the extent of those modifications on the proteins leaving the bioreactors.

In the last step of the above approach for the design of an RTRT strategy, the use of UO interaction studies and plant-wide simulation enables the evaluation of the potential effects of disturbances and uncertainties on the CQAs. The effects of disturbances propagate from one unit operation to the next, and connecting the UO models together enables the evaluation of the effects of this propagation on the CQAs of the final product. Such a strategy based on a plant-wide simulation has been successfully demonstrated in the end-to-end continuous manufacturing of tablets of a direct renin inhibitor, aliskiren hemifumarate, which is detailed by Lakerveld et al. (2015). The implementation of this strategy for both small molecules and a biopharmaceutical manufacturing platform is discussed in a later section.

The above steps in the systematic approach for the design of the overall RTRT strategy apply irrespective of whether the unit operations and plant are at the laboratory or production scale. If multiple scales are employed, then all of the steps including model validation would first be applied at the laboratory scale where experiments are cheaper and faster to carry out, and then technology transfer would be to the production scale. Scale-up is facilitated by employing the same modeling and control platforms at different scales.

Four RTRT strategies for ensuring the satisfaction of a particular CQA specification are (Myerson, Krumme, Nasr, Thomas, & Braatz, 2015):

1. Direct measurement of the CQA during manufacturing,
2. Prediction of the CQA based on a mechanistic model that is fed measurements of related variables and is running in parallel with operations,
3. Prediction of the CQA based on an empirical or semi-empirical model (e.g., response surface map, PLS model) that is fed measurements of other variables, and
4. Operation of the critical process parameters (CPPs) to lie within a design space, that is, some specified combination of variables shown in offline studies to provide assurance of product quality.

The first three strategies can be used in feedback and feedforward control, while the last strategy is only feedforward control. The strategies can also be combined, to provide redundancy, and/or can be combined with end-product testing.

Feedback control that makes use of a reliable direct real-time measurement or prediction of the CQA enables increased assurance of product quality, and so is preferable for real-time release. The feedback controller can be designed to shift a measured CQA toward its most desired value, and so reduces variation from any target value for the CQA. If the CQA can be predicted in real time accurately and reliably using a mechanistic, empirical, or semi-empirical model, then that prediction can be used in place of the direct measurement in a feedback control strategy. A CQA is more tightly controlled when feedback control is used, as the use of feedback provides the capability to force the CQA to converge to a target value. Feedforward control enables estimates of upstream material attributes to be used to improve the control of downstream material attributes, which is especially useful for batch processing and continuous-flow processes with long residence times. Usually the best performing control systems based on the first three strategies use a combination of feedback and feedforward control. Detailed comparisons of feedback, feedforward, and combined feedforward-feedback control in pharmaceutical manufacturing have been published (Lakerveld et al., 2015).

1.2 | Quantifying critical quality attributes: State of the art and analytic challenges

The first strategy for ensuring the satisfaction of a particular CQA specification described in the previous section uses the direct measurement of the CQA during manufacturing. Direct in-process measurement of CQAs falls under the umbrella of process analytical technology (PAT). PAT is defined by the FDA as "a system for designing, analyzing, and controlling manufacturing through timely measurements of critical quality and performance attributes of raw and in-process materials and processes, with the goal of ensuring final product quality" (FDA, 2004). The reliability of the measurements used in a proposed RTRT strategy is critically important, as end-product testing is not allowed to replace a failed RTRT (EMA, 2012). As such, this section also discusses risks associated with implementing direct in-process CQA measurements as part of RTRT.

Some common CQAs for monoclonal antibodies are aggregates, clipped forms, low molecular weight impurities, post-translational modifications (e.g., oxidation, deamidation, and glycation), sequence variants, charge heterogeneity, N-glycosylation (e.g., occupancy, glycoform, galactosylation, fucosylation, sialylation), process-related impurities, viral contaminants, bioburden, sterility, endotoxin, binding to target, and Fc receptor binding. To enable RTRT, analytical technologies ideally should require limited sample handling (such as purification steps), provide timely results, and be able to be qualified and validated. The ideal technology should be fully automatable, fast relative to the process dynamics, and robust. The degree to which the current technologies are able to meet these criteria varies depending on the CQA. Data collected via RTRT are not only valuable for quality assurance of end product, but also provide information to the control system to improve operations.

Table 1 summarizes the state of the art for direct CQA measurements using monoclonal antibody CQAs as examples. Given that the focus of this article is on RTRT, of the many measurement technologies, only the gold-standard and emerging techniques are presented. Gold-standard techniques are defined as providing robust measurements, currently most widely accepted, but are not necessarily ideal for RTRT. Emerging techniques, presented in italics, are defined as being very promising for meeting the requirements of RTRT but are not fully developed. Prior to implementation on the floor, these emerging techniques will need to be validated to deliver the required accuracy, precision, and robustness to meet the current good manufacturing practice (cGMP) requirements.

Sample preparation is an important consideration for analytical techniques (Konstantinov & Cooney, 2015). Either automating the sample preparation step or replacing these analytical techniques with methods that do not require sample preparation would facilitate major progress for real-time monitoring. Measurements are typically described as either in-line, on-line, at-line, or off-line. In-line measurement is the most desirable for RTRT because no sample is removed from the process stream, which implies no sample preparation is required and measurements can be made quickly. An on-line measurement requires samples to be diverted from the process stream, but may be returned. At-line measurement requires samples to be removed from the process but analysis can be performed nearby, whereas off-line measurements must be performed in separate facilities. When sample preparation only involves physical separations, such as in SEC, there is potential for on-line process integration. When reactions or a series of complex steps are involved, as in LC-MS, replacement technologies may be a better fit. Automated sample handling and chip-based technologies are two approaches for simplifying sample preparation.

At-line analysis, enabled by automated sample handling, is an intermediate approach between fully automated and manual sampling (Konstantinov & Cooney, 2015). The combination of at-line measurement with automated sampling results in a combined approach that is on-line. When using automated sampling, ensuring representative sampling is of key importance. For all applications of automated sample handling, the volume of samples should be small to avoid product loss but large enough that the product can be accurately characterized. Currently, only a few companies offer automated sample handling for manufacturing. Seg-Flow, a technology from Flownamics, is one such technology which is able to perform on-line sampling from bioreactors and perform sample delivery to up to four analyzers and/or fraction collectors. The sample line is cleaned and sterilized after every sample, and the in-situ sampling probe is cGMP validated.[2] The BaychroMAT Process from Bayer Technology Services and Modular Automated Sampling Technology (MAST) from Bend Research are two other options that allow for collection of sterile bioreactor samples. Both technologies can be used in cGMP manufacturing settings. Bend Research reports that it is working to integrate its technology with additional instruments.

Several companies are developing chip- or cartridge-based systems for PAT. These platforms are often able to decrease the quantity of sample required and/or the amount of sample preparation. For example, the Peggy Sue from ProteinSimple is able to provide information about charge heterogeneity using very small volumes (5 µl) directly from cell culture supernatant samples without protein purification, compared to other chip-based separation techniques such as GXII from Perkin Elmer. Peggy Sue is not fully automated and requires manual sample loading and data analysis. Cartridge-based systems are becoming available for assays such as the Charles River PTS™/MCS™ cartridges, which come pre-loaded with LAL reagents. These tests can be performed in 15 min. Table 1 has a complete discussion of the technologies.

1.3 | Quantifying critical quality attributes: Operational challenges

Analytical technologies for enabling RTRT also face several operational challenges. The potential failure of instruments is a risk that can be mitigated by having duplicate or backup instruments. Potential drifts in instruments should be characterized. Procedures must be implemented to ensure that recalibration occurs at a rate such that the associated CQA measurements remain accurate. In combination with the accurate and timely measurement, availability of control levers for

TABLE 1 Gold-standard and emerging technologies available for measurement of a subset of monoclonal antibody CQAs in upstream and downstream processing

CQA	Measurement technology and description
Aggregates (high molecular weight impurities)	Size exclusion chromatography (SEC) is a separation based on size and shape with detection via UV fluorescence or light-scattering detectors. SEC is potentially an online method with 5–10 min turnaround, but samples must be purified before analysis.
	Slanted nano-arrays (SNA) are a chip-based technology that uses angled nanofilters for size-based separations. Detection is via fluorescence. SNAs operate continuously and have very low limits of detection (Ko & Han, 2014; Ko et al., 2015; Ouyang, Ko, Wang, Hancock, & Han, 2015). *This emerging technology has not yet been validated for RTRT purposes.*
	The combination of spectral analysis and the partial least squares (PLS) chemometrics technique has been demonstrated to be a promising for quantification of protein impurities. By combining diode arrays for detection and PLS for analysis, co-eluting proteins could be analyzed online (Brestrich, Briskot, Osberghaus, & Hubbach, 2014; Brestrich et al., 2015; Hansen, Skibsted, Staby, & Hubbuch, 2011; Kamga, Lee, Liu, & Yoon, 2013).
Clipped forms and low molecular weight impurities	Sodium-dodecyl sulfate capillary electrophoresis (CE-SDS) is a gel technique that uses electrokinetic separations. Detection is often via UV. CE-SDS is automatable, quantitative, and robust (Rustandi, Washabaugh, & Wang, 2008). Chip-based options are also available. These features make it very desirable for RTRT PAT.
Post-translational modifications (oxidation, deamidation, glycation) and sequence variants	Liquid chromatography mass spectrometry (LC-MS) is a hyphenated technology to first separate digested proteins (LC) then analyze the peptides (MS) (Mann & Jensen, 2003). LC-MS provides quantitative information but requires extensive sample preparation and analysis. Shortening the procedure as well as automating the data processing are the recent trends for LC-MS method development. Currently few other techniques are able to produce a similar quality of data.
	Top-down and middle-down LC-MS method by direct analysis of protein biopharmaceuticals with minimized sample preparation may represent a future trend, which will provide direct information related to the proteoforms rather than extracting information from peptide fragments from the proteins (Bush, Zang, Belov, Ivanov, & Karger, 2016; Fornelli, Ayoub, Aizikov, Beck, & Tsybin, 2014; Moradian, Kalli, Sweredoski, & Hess, 2014).
	ZipChip™ is an emerging technology developed by 908devices. ZipChip utilizes microfluidic technology to quickly perform sample preparation which then can be utilized directly for MS (Redman, Batz, Mellors, & Ramsey, 2015).
Charge heterogeneity (acidic and basic isoforms)	Ion-exchange chromatography (IEC) is separation driven by protein surface charge difference. IEC is an established technology.
	Imaged capillary isoelectric focusing (icIEF) is a capillary-based pI-driven separation. Detection is done using continuous UV scanning of the capillary. This approach avoids the mobilization step of traditional cIEF, which greatly decreases runtimes. Typically, icIEF can be performed in twenty minutes. The major drawback of icIEF for RTRT is that sample preparation is required (Michels, Salas-Solano, & Felten, 2011; Sosic, Houde, Blum, Carlage, & Lyubarskaya, 2008).
	Peggy Sue and NanoPro 100 (ProteinSimple) offer a solution to measure charge heterogeneity of protein in complex matrix without purification. Using the devices, proteins are first separated by charge (or size), then immobilized on the surface of the capillary wall. The proteins are next recognized by affinity reagent and detected using a chemiluminescent substrate. The potential to directly analyze non-purified process samples is the main benefit of these techniques.
N-glycosylation (occupancy, glycoform distribution, galactosylation, fucosylation, sialylation, fucosylation, sialylation, etc.)	Hydrophobic interaction chromatography (HILIC) is a chromatography technique which uses a polar stationary phase and a mobile phase with a high percentage of acetonitrile. HILIC is well suited to glycan analysis because of its high detection sensitivity, good reproducibility and its ability to separate positional isomers. Its main disadvantage for RTRT is that the sample preparation is time-

(Continues)

TABLE 1 (Continued)

CQA	Measurement technology and description
	consuming (Ahn, Bones, Yu, Rudd, & Gilar, 2010).
	Label-free lectin microarrays are a technology that uses HIS-tagged affinity reagents in a 96-well plate and NIR detection (Salem, Nelson, Kim, & Strano, 2016). *This emerging technology is a fast and efficient approach to glycoprofiling but will take time to validate for RTRT purposes.*
	Aptamer-based biosensors are another emerging technology for the analysis of n-glycans. Aptamers are nucleic acid sequences which a selected for their affinity for small molecules, proteins and cells. These biosensors are combined with gold-nanoparticles as nanoprobes to achieve very low limits of detection (Chen et al., 2014). *Like label-free lectin microarrays, this approach will take time to validation for RTRT purposes.*
Viral contaminant	In vitro cell culture is the gold standard test for determining the presence of contaminants, and is recommended by ICH Q5 (ICH, 1997). Its major drawback is that it is very time consuming with a recommended incubation of 28 days.
	Next-generation sequencing (NGS) is a non-Sanger-based sequencing technology that is faster than previous techniques (Schuster, 2008). *NGS is promising for the future because of its speed, but will take time to validate for RTRT purposes.*
Microbial contaminant (bioburden and sterility)	A microbial culture test reporting count of colonies is the current standard method for bioburden and sterility. This technique is a bottleneck to RTRT because the growth assay is slow. Companies such as Charles River are attempting to build devices that cut the test time down from days to minutes. The Charles River device is currently marketed for titer verification prior to inoculation and not as release testing.
	Polymerase chain reaction (PCR) is a DNA amplification technique that is coupled with electrophoresis for detection. It is easy, fast, and cheap compared to cell culture but needs specific primers (i.e., is not reagentless). Therefore PCR is only suited to the case where there are specific contaminants of interest.
	NGS, as described above, could also be applied to bioburden and sterility. NGS is reagentless but requires intensive analysis.
Endotoxin	The limulous amoebocyte lysate (LAL) test is the most popular method for endotoxin testing. LAL, which is derived from the blood cells of horseshoe crabs, clots in the presence of endotoxin. LAL tests are fast, inexpensive, and easy to run compared to other available methods. Some companies are pursuing cartridge-based assays which may reduce test times even further.
Binding to target and Fc binding	ELISA (enzyme-linked immunosorbent assay) is a binding affinity assay in which detection is enabled by specific antibody-antigen interactions. ELISA is typically performed using a 96-well plate format and is easy, fast, and cheap, in part because of its ability to be automated using robots.
	Surface plasmon resonance (SPR) is an optical method for detecting molecular interactions (Pattnaik, 2005). Like ELISA assays, this method requires affinity reagents. ELISA is generally preferred to SPR because it is higher throughout, user-friendly, and can be multiplexed.
	AlphaScreen (amplified luminescent proximity homogenous assay) is a microplate-based test that utilizes bead-based chemistry. AlphaScreen uses a luminescent/fluorescent signal to measure binding. The technology is fast and easy to use (PerkinElmer, 2016).
	FRET (fluorescence resonance energy transfer) is a distance-dependent interaction which measures molecular interactions via fluorescent detection (Constantinou & Polizzi, 2013). Several companies have come out with technologies that utilize FRET including LANCE TR-FRET.
	Octet is a well microplate based system for measuring biomolecular interactions. Octet uses a proprietary technology to perform label-free, high-throughput, and real-time measurements.
Process-related impurities	Immuno-affinity assays (e.g., ELISAs as described above) is typically used for

(Continues)

TABLE 1 (Continued)

CQA	Measurement technology and description
	protein-based impurities such as residual HCP and Protein A.
	Liquid chromatography (LC) followed by spectroscopic detection (such as UV, fluorescence, or a universal detector such as a corona charged aerosol detector). This technique is typically used for non-proteinaceous impurities.
	Mass spectrometry (MS) is a technique that has increased use in monitoring proteinaceous impurities (Gülbakan, Barylyuk, & Zenobi, 2015; Oedit, Vulto, Ramautar, Lindenburg, & Hankemeier, 2015). *MS is desirable because it is sufficiently sensitive to meet detection limits, and provides specific information of the impurity, such as HCP.*
Protein concentration	UV detection of protein is a classic method. Its benefit is that it is non-destructive and fast. Its need for very precise calibration is a major drawback (Lonza, 2009). UV testing is not done online and is being replaced by FlowVPE (see below) for RTRT applications.
	Refractive index (RI) is a classic method for measuring protein concentration that is non-destructive but the result is highly dependent on the buffer solution, which limits its application.
	SoloVPE is an assay from C Technologies, Inc. which provides fast and accurate concentration measurements. SoloVPE utilizes variable path length technology which measurements to be performed without dilution and baseline correction. Recently the FlowVPE has been introduced which would allow for online concentration measurements (Huffman, Soni, & Ferraiolo, 2014).

The emerging technologies are italicized. Sensor technologies for non-CQAs such as pH, temperature, pressure, flow rates, and osmolality that are important in operations and control systems design are not included. Also not included are technologies for measuring CQAs associated with formulation, fill, and finish, such as color, appearance, and particles.

Additional information sources for Table 1 are:

(1) *ZipChip*. 908devices, Boston, Massachusetts, http://908devices.com/products/zipchip/, accessed May 9, 2017.

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(12) *An Overview of Slope Spectroscopy & the SoloVPE*, C Technologies Inc., Bridgewater, New Jersey, http://www.solovpe.com/sites/default/files/literature/SoloVPE%20Abstract.pdf, accessed on February 14, 2017.

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the CQAs also determines the effectiveness of a PAT tool. Lack of a real-time control lever for the CQA does not remove the benefit of the measurements in process. A CQA deviation detected earlier can induce an investigation and prevent this deviation from occurring in subsequent lots.

Automated sampling is one technology that begins to bridge the gap between analytical advances and operational implementation. An important benefit of automated sampling technologies is the ability to maintain sterility in the product stream. Sterility is important for biopharmaceutical manufacturing: microbial and viral contaminants can cause a loss of drug potency, change impurity profiles, increase in the level of bacterial endotoxins, and result in production shutdowns and manufacturing delays (Suvarna, Lolas, Hughes, & Friedman, 2011). In recent years, the ability to ensure sterility and prevent contamination has greatly benefited from single-use, disposable components (Shukla & Gottschalk, 2013). Automated sampling has the potential to

maintain a fully closed system and therefore decrease the risk of contamination even further.

The current tests for viral and microbial contamination take days and are the bottleneck of analytical technologies for RTRT. At this time, viral and microbial contaminant release testing must be carried out on the final drug product (ICH, 1997). Validating viral and microbial contaminant clearance as part of an RTRT strategy is a significant challenge. Some work has been reported on the use of next-generation sequencing (NGS) to accelerate the test for contamination.[3] NGS is desirable over other technologies such as PCR because it has the ability to screen for all potential pathogens. When applying customized primers, NGS is can target monitoring specific pathogens as well. However, NGS requires complex data analysis and therefore further development of the technology is needed.

1.4 | Promising cases of RTRT for biopharmaceuticals

The biotechnology industry has made progress in recent years in the exploration of PAT for advanced process control and RTRT of biopharmaceuticals through several avenues. Some of the attempts in RTRT have been published and include one or more of three elements, with corresponding RTRT strategies in parenthesis: product/process understanding (Strategies 2 and 4), in-process parametric control (Strategies 2–4), and attribute testing at an earlier step in the process (Strategies 1 and 3) (EMA, 2012).

The proposed RTRT approaches commonly start from identification of critical control points (CCPs) of the product quality attributes (PQAs) and the critical process parameters as well as input parameters at the control points that drive the formation and level of the PQAs. PQAs refer to the complete list of quality attributes of a product and include CQAs as well as non-CQAs. A CCP of a PQA refers to a particular unit operation of a production process, after which there is little to no potential for a PQA to change (WHO, 2003). Applying a validated advanced process control for a PQA at its CCP, utilizing real-time product quality prediction through modeling or direct measurement and allowing self-correction of the process to meet the desired range of the PQA, represents one of the approaches for RTRT. In Table 2, CCPs are identified for different PQAs for typical fed-batch monoclonal antibody drug substance and filter-and-fill drug product manufacturing processes. A few promising applications described below are also mapped in Table 2.

Control of the glucose feeding based on real-time monitoring of the culture at the bioreactor stage using a Raman probe allows a more precise control of the glucose concentration in the culture than conventional periodical glucose measurements and thereafter a more consistent level of glycation in monoclonal antibody products manufactured in Biogen, which provides an avenue for RTRT of glycation (Berry et al., 2016). Similarly, Amgen reported real-time glycosylation monitoring of bioreactor using micro-sequential injection system coupled with UPLC N-glycan analysis (Tharmalingam, Wu, Callahan, & Goudar, 2015). Although the report mainly advocated use of the method for process monitoring and control, the method can also be used for RTRT, provided that the N-glycosylation remains constant after the bioreactor step (CCP). In another report, Amgen showcased direct control of high-mannose N-glycosylation levels by controlling mannose feed to the bioreactor (CCP) (Zupke et al., 2015). This procedure was enabled by combining near real-time mass spectrometry measurements and standard bioreactor monitoring with a nonlinear predictive model, developed based on understanding of the relationship between bioreactor mannose concentration with high-mannose level on mAb and bioprocess performance. The authors commented that this approach of ensuring that product quality falls into the targeted range through active control with direct real-time PQA measurement can potentially enable RTRT of the PQA. Biogen replaced traditional chromatography assays by multivariate models that predict lower-pI isoforms and N-glycan properties, for example % galactosylation and %sialylation, based on cell culture process parameters, as part of the drug substance and drug product release of a commercial mAb that received approval by the EMA in 2016. This approach successfully removed the end-product release testing of these product qualities and provided an avenue for RTRT using product quality predictive models established based on process understanding. In similar way, Sandoz scientists have demonstrated prediction of multiple PQAs based on commonly available process parameters using modelling termed as *performance-based modeling* (Schmidberger, Posch, Sasse, Gulch, & Huber, 2015).

For the PQAs lacking mature control loops, moving the analytical measurements earlier in the process, such as real-time at (or soon after) the CCP has been a second approach explored by the biotechnology industry. A few companies are in the process of filing for RTRT with multiple regulatory agencies for their commercial products relying on rich product and process experience gathered from years of manufacturing, in-process testing, and release testing of drug substance (DS) and drug product (DP). For example, pH and osmolality, originally part of release testing of DS and DP, can be replaced by testing of the prime/rinse buffer at the UF/DF step which can determine the formulation buffer of DS and DP. Protein concentration determination can also be moved to the UF step by testing the UF pool instead of testing during DS and DP release (assuming a simple filter-and-fill DP process). Some repetitive testing of PQAs was successfully removed from the DS step and only performed at DP release (Cherney, 2016). Removal of tests for process-related impurities such as host-cell protein, DNA, and ProA leachate is common when clearance capability of the process is demonstrated using spiking studies. Amgen reported a multi-attribute method (MAM) that can potentially replace multiple traditional assays such as ion-exchange chromatography for charge variants, sodium-dodecyl sulfate capillary electrophoresis (CE-SDS) for purity and impurities, identity, and some process-related impurities (Rogers et al., 2015). The MAM method has been filed as a release testing method for investigational products and can be potentially performed in the process rather than for DS or DP.

1.5 | What can be learned from RTRT for small-molecule and protein pharmaceuticals

The potential of RTRT has been demonstrated in both academic research laboratories and industrial practice. One example is the

TABLE 2 Identifying the CCPs for quality attributes for a typical monoclonal antibody process.

	DS upstream		DS downstream				
Quality attribute	Raw material	Bioreactor	Affinity capture	Charge	Hydro-phobicity	UF/DF and DS fill	Drug product
N-glycan	CCP	CCP					
High mannose N-glycan		CCP (real-time product attribute control, Zupke et al., 2015)					
Glycation		CCP (real-time feed control using Raman probe, Berry et al., 2016)					
Charge variants				CCP			
High molecular weight (HMW) species					CCP	CCP	CCP
Process-related impurities (e.g., Protein A leachate, host-cell protein, DNA)		CCP, "validate-out" approach; remove test using process clearance validation with spiking studies					
Excipients (e.g., PS80)						CCP	
Protein concentration						CCP	CCP (if involving dilution during DP manufacturing)

MIT-Novartis end-to-end continuous small-molecule pharmaceutical pilot plant where mathematical modeling predicted that all CQAs would be satisfied before the plant was constructed, which was then confirmed after the plant was constructed and operated (Gefroh & Lutz, 2014; Jiang & Braatz, 2016). The satisfaction of the CQAs for this particular application was primarily assured by feedback control systems designed based on simplified mechanistic models, with some use of feedforward control and empirical modeling of low-concentration impurities. The mathematical model of the entire operations was highly effective in predicting the product CQAs and evaluating the plant-wide control strategy, and so is suitable for RTRT application. The evaluation of the overall RTRT strategy in plant-wide simulation would increase confidence and reduce risk associated with various types of disturbances and uncertainties, whether manufacturing a small-molecule pharmaceutical or a biologic drug product.

An example of a successful RTRT regulatory submission is the manufacturing of a small-molecule drug product by Bristol-Myers Squibb (BMS) (Singh, 2015). The BMS regulatory submission included the application of PAT for in-process control and RTRT, quality risk and knowledge management plans, and QbD/PAT-based training of site personnel. The endpoints for two blending processes and the potency and content uniformity of the drug product (the CQAs used in RTRT) were determined by near-infrared (NIR) spectroscopy, with appearance, disintegration/dissolution, and high-pressure liquid chromatography (HPLC) handled by end-product testing. The blending endpoint was determined using a design space strategy, whereas the RTRT strategy was by direct measurement of the CQA. EMA and FDA approved the RTRT filing based on process development and production data comparing on-line tablet NIR with off-line HPLC (Singh, 2015). BMS provided a decision tree that specified when on-line tablet NIR could be used in place of end-product testing by HPLC.

An integrated and scalable cyto-technology system (InSCyT) to produce biologic drugs (e.g., human growth hormone, interferon−α2b) was recently developed at MIT (Lu et al., 2015). The approach for designing the overall control strategy adapts and extends a strategy used in the end-to-end continuous manufacturing of chemicals and small-molecule pharmaceuticals (Lakerveld, Benyahia, Braatz, & Barton, 2013; Mascia et al., 2013; Nagy & Braatz, 2012). Namely, the plant-wide control strategy is designed systematically by a modular approach in which UOs are interconnected in a plug-and-play manner within a plant-wide simulation. Mechanistic models are developed for each unit operation in the biopharmaceutical manufacturing platform, with the mechanistic models augmented by empirical models for the prediction of CQAs for which mechanistic understanding is not yet well developed, such as for post-translational modifications. The design space strategy is used to assure that very low concentration impurities, for which neither empirical nor mechanistic models are available, are within specifications. Numerical algorithms are used that robustly simulate the nonlinear continuous and discrete operations that arise during the startup, shutdown, and intermediate operations of the biopharmaceutical manufacturing. Such plant simulation technology enables the evaluation and optimization of measurement, control, and RTRT strategies during all operational modes of the biopharmaceutical manufacturing plant. In addition to the three strategies for ensuring the satisfaction of a particular CQA specification discussed above,

advances in sensor technologies will enable greater use of the first of the four strategies—direct measurement of the CQA during manufacturing.

1.6 | Predicting the Growth of RTRT

RTRT would be facilitated by advances in sensor technologies, which show good promise for micro- and nanosensors based on spectroscopies such as Raman, UV/Vis, and fluorescence. Response surface and multivariable statistical models constructed by ordinary and partial least squares and related methods are already well established in the biopharmaceutical industry (Lakerveld et al., 2013; Nagy & Braatz, 2012; Severson et al., 2015) and can be enablers of RTRT. Often, these techniques are combined with data from design of experiments to improve understanding of the CPPs (Agarabi et al., 2015, 2017). More recently, machine learning methods that construct spare models have been shown to produce more accurate predictions than the methods commonly used in the biopharmaceutical industry (Severson et al., 2015), which could further enable RTRT applications.

RTRT would also be facilitated by more powerful software systems that incorporate a data historian that supports design of experiments, parameter estimation, and quality assurance; an optimizer of operations that is flexible enough to handle a combination of batch/semibatch recipes and (semi-)continuous unit operations; and control strategies that optimize quality while automatically rejecting any off-spec product.

2 | CONCLUSIONS AND FUTURE PERSPECTIVES

RTRT has the potential to increase quality assurance, improve productivity, and reduce cycle times, with associated potential benefits to the biopharmaceutical manufacturing field and to patients. However, RTRT must be implemented with care, as a failed RTRT test cannot be replaced by a successful end-product test. The keys to enabling RTRT lie in integrated sensor technologies, mathematical models, and control strategies. These technologies require feedback and consensus from regulatory agencies for their use and can provide a high degree of assurance that continuous drug supply is available to patients. While the implementation of RTRT to biologic drug manufacturing is challenging, its value has already been demonstrated in the small-molecule pharmaceutical industry. To implement a complete RTRT solution, biologic drug manufacturers need to consider the special attributes of their industry, particularly sterility and the measurement of viral and microbial contamination. Continued advances in on-line and in-line sensor technologies are key for the biopharmaceutical manufacturing industry to achieve the potential of RTRT.

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ENDNOTES

[1] As discussed by Lakerveld et al. (2015), Mascia et al. (2013), and Myerson et al. (2015), integrated manufacturing (aka computer-integrated manufacturing) is the use of computers to control the entire production process, which allows data collected on individual processes to be collectively used to optimize plant operations.

[2] This information comes from the below sources: (i)*BaychroMAT Process Analysis System*, Roche Innovatis AG, Bielefeld, Germany and Bayer AG, Leverkusen, Germany, http://www.bayertechnology.com/en/solutions/operation-support-safety/process-analysis-technology/baychromat.html, accessed February 14, 2017; (ii) *Modular Automated Sampling Technology (MAST)*, Bend Research, Bend, Oregon, http://mastsampling.com, accessed February 14, 2017; (iii) *Seg-Flow Automated On-Line Sampling Solutions*, Flownamics, Madison, Wisconsin, http://www.flownamics.com/automated_bioreactor_sampling_Seg-Flow.html, accessed February 14, 2017.

[3] Information resource: E. Toso, C. Modena, and F. La Neve, *Next-generation Sequencing Used for Biological Quality Control in Biopharma Production*, Illumina, San Diego, California, September 2014, available at https://www.illumina.com/content/dam/illumina-marketing/documents/icommunity/article_2014_09_merck_bqc.pdf

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