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Towards real-time monitoring of therapeutic protein quality in mammalian cell processes

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Protein biopharmaceuticals are mainly produced in mammalian cells which can perform human-like post-translational modifications crucial to protein function. Subject to high variability, these critical quality attributes should be monitored and controlled during the manufacturing process. However, the large time requirements for analysis have been a bottleneck. Recent advances towards automated and high-throughput techniques, combined with multivariate data analysis, are increasingly providing relevant process knowledge in near real-time. New or re-designed analytical tools suited for monitoring product quality are starting to fit in this landscape. Moreover, *omics* technologies are expanding our understanding of how intracellular mechanisms and the extracellular *milieu* influence protein quality and quantity, reshaping the adoption of Process Analytical Technology (PAT) and Quality by Design (QbD) in the biopharmaceutical industry.

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Introduction

Recombinant proteins, including monoclonal antibodies (mAbs), have reached a market value over $100 billion and represented 32% of the new drugs approved by the FDA in 2011 [1,2]. The number of currently approved mAbs is 28, with 25 more in late stage clinical trials, demonstrating the growth of this class of proteins which is forecasted to account for 50% of the top selling drugs during the current year [2]. From all the host cells used to produce therapeutic proteins, mammalian cells have emerged as preferred expression systems due to their ability to perform human-like post-translational modifications (PTMs) [3]. Chinese Hamster Ovary (CHO) cell lines, in particular, are used in the manufacture of 60–70% of marketed biotherapeutics [4•]. It is crucial to produce these biotherapeutics correctly processed such that they can display the desired functionality *in vivo*, without triggering unforeseen secondary effects or undesired interactions inside the human body. As biopharmaceutical production is intrinsically subject to variability, continuous monitoring of product quality attributes and ideally all process parameters which impact product quality is desirable, to allow timely in-process corrections when any undesired protein characteristic is detected. However, methods for online monitoring of protein quality attributes are still in their early days; improving the robustness and accuracy of current analytical technologies, while simultaneously reducing the time required for analysis, is important to increase efficiency in the biomanufacturing industry and meet increasingly tight quality policies from regulators. Moreover, increasing process knowledge through data collection is essential to find the key process variables that have an impact on protein attributes. These trends could have the fullest expression in the growing landscape of biosimilar (follow-on) products, where substantial drive and opportunity exist to profit from these methods to better characterize and guarantee similarity of high-value therapeutic proteins (Box 1). Here, we overview the efforts undertaken towards online monitoring of quality-related protein characteristics and cover relevant analytical methods and chemometric tools used to correlate measurable process variables with important quality attributes or with the key process parameters which impact them. The process information thus extracted builds our ability to operate biopharmaceutical production under the Quality by Design (QbD) and Process Analytical Technology (PAT) initiatives (Box 2).

Protein quality analysis

The product characteristics that impact its safety and efficacy, defined as critical quality attributes (CQAs) (Box 2), should be maintained within an appropriate range to meet the desired clinical performance [5]. Some product contaminants can significantly impact the functionality and safety of the recombinant protein *in vivo*, including alterations on the different levels of protein structure, such as amino acid deamidation, oxidation or sulfation, cross linking, disulphide bonds, and cleavage of peptide fragments [6]. Typical impurities are host cell DNA or proteins and raw materials from the production

Box 1 Biosimilars

Biosimilars can be roughly defined as the generics of biopharmaceutical products. They possess the same amino acid sequence of approved therapeutics, but as the manufacturing process changes (including producer cell clone), concerns on whether the biosimilar maintains the clinical profile of the reference product are raised. Whilst for small-molecule drugs the approval process for generic versions can be abbreviated (being sufficient a demonstration of pharmaceutical and biological equivalence), due to the size and complexity of protein therapeutics it is not simple, if even possible, to demonstrate complete equivalency to a reference product. To regulate biosimilars development, it is important to define how much and what kind of data is needed to establish clinical comparability [7•]. The FDA has determined that PTMs, aggregation level and the 3D structure of a protein biosimilar must be comparable to the reference product [7•]. After quality validation of a biosimilar, it is important to demonstrate that CQAs are maintained within the product design space throughout the production process, as changes can happen very easily due to batch-to-batch variability, process drifts (which gradually lead the process towards changes in the product) and manufacturing changes [9]. Correlations between measured protein characteristics and clinical performance would further ease the regulatory approval and lower development costs of biosimilars.

process. Additionally, in the context of biosimilar production (Box 1), regulatory agencies demand comparability studies to the original biopharmaceutical in terms of post-translational modifications (PTMs, in particular glycosylation) as well as aggregation and three-dimensional (3D) structure [7•]. These characteristics are focused in more detail in this section, where we highlight recent developments towards faster and simpler methods for monitoring quality-related protein attributes during bioproduction.

Box 2 QbD, design space and PAT

Quality by Design (QbD) is an initiative promoted by the regulatory authorities encouraging biopharmaceutical companies to build processes designed to yield consistent product quality instead of relying solely on final testing of product batches (quality by testing) [55]. A thorough understanding of the process, the product and every variable affecting both is needed. Considering the high number of necessary experiments, measurement times and sample throughput capacity of current analytical technologies, evaluating all combinations of process parameters is time-consuming and labour-intensive. To this end, Design of Experiments (DoE) can be used to simulate all possible interactions between process variables and to prioritize those worthy of further investigation, substantially reducing the experimental burden. This will help to define the range of each critical process parameter (CPP) which assures the desired CQA profile — process design space. Finally, the CPP should be monitored and controlled to ensure that the process is operating within the design space, acknowledging the Process Analytical Technology (PAT) initiative [5]. PAT can be defined as a set of tools and technologies devised to analyse, monitor and ultimately control bioprocess key characteristics, preferably in real-time and in different stages of the process, for early fault detection and correction, allowing efficient control of process outcomes (e.g. product quality) and facilitating the path for approval. Biopharmaceutical companies adopting and implementing these initiatives will benefit from regulatory flexibility, with reduction of post-approval submissions, as it is not considered a ‘change’ if a CPP is altered within the design space [5,55].

Glycosylation, the addition of glycan structures to polypeptide chains, can influence the physico-chemical properties (e.g. folding, solubility, electrical charge, stability) and the clinical function (e.g. efficacy, *in vivo* half-life, immunogenicity, antibody-dependent cellular cytotoxicity (ADCC) and complement-dependent cytotoxicity (CDC)) of therapeutic proteins [8]. It is one of the most sensitive quality-related attributes and contributes extensively to the heterogeneity of the protein mixture obtained at the end of a typical process due to slight differences in sugar sequences and site occupancy. Incidentally, glycosylation profiles can be affected by the producer cell line, culture conditions (dissolved oxygen, nutrients, osmolality), mode of operation (batch, fed-batch or perfusion) and the downstream processing [9–11]. Even slight changes in the overall production process may translate in significant differences in glycan profiles, for which reason analytical testing is essential to demonstrate that the main characteristics of the product are maintained throughout the process within acceptable ranges.

Traditional sample preparation and glycosylation analysis is time-intensive and labour-intensive, involving glycan release from proteins, enzymatic digestion and subsequent labelling or derivatization. Samples then undergo separation (often using chromatography-based methods) followed by analysis, usually through mass spectrometry (MS) [8,12]. Novel methods to assort glycosylation and other CQAs of therapeutic proteins need to be developed, specially to allow real-time determination and automation, while maintaining or improving the accuracy and sensitivity of existing methods. As an example, a recent method for profiling N-glycosylation based on fluorescent labelling and ultra-performance liquid chromatography (UPLC) was developed, taking five hours to analyse the glycan composition of mAbs directly from the cell culture supernatant [13•]. A similar method with slight differences in sample preparation (using a different labelling dye and a higher temperature for the *N*-glycan release step), could perform the same analysis in less than 90 min [14]. Both methods were developed in a 96-well plate format, enabling high-throughput analysis. Another rapid and automated method to distinguish glycosylation patterns of therapeutic antibodies has been developed at the microfluidic scale [15]. During manufacturing, other enzymatic and chemical PTMs such as sialylation, deamidation, C-terminal lysine amidation, galactosylation and tryptophan oxidation contribute to high batch-to-batch heterogeneity in charge variants of mAbs that can affect their pharmacokinetics [16••]. Aiming at near real-time monitoring of this important attribute, a 2D-high-performance liquid chromatography (2D-HPLC) method was developed to

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