Biologics, such as therapeutic monoclonal antibodies (mAbs), are complex protein molecules produced from mammalian tissue culture cells through recombinant DNA technology. As a result of naturally-occurring molecular heterogeneity as well as chemical and enzymatic modifications during manufacture, process, and storage, there are many product quality attributes (PQAs) present in therapeutic proteins. These PQAs can potentially include: product-related structural heterogeneity related to glycosylation profile, disulfide bond pattern, and higher order structure; product-related degradants and impurities, such as deamidation, oxidation, sequence variants; and process-related impurities and residuals, such as host cell protein (HCP), host cell DNA, and residual protein A [1]. Regulatory agencies have recently recommended a Quality by Design (QbD) approach for the manufacturing of therapeutic molecules [2-5], which requires in-depth understanding of these PQAs at the molecular level to ensure that the drug products meet the desired safety and efficacy profiles [6]. The QbD guidelines require development of a quality target product profile (QTPP) that identifies critical quality attributes (CQAs) and implementation of control strategies to ensure that the QTPP is achieved. QTPP is a prospective summary of the quality characteristics of a drug product to be achieved to ensure the desired quality, safety and efficacy [2]. QTPP describes the design criteria for the product and forms the basis for determination of the CQAs, critical process parameters (CPPs), and control strategy. A CQA is a physical, chemical, biological, or microbiological property or characteristic that should be within an appropriate limit, range, or distribution to ensure the desired product quality [2]. A CQA is identified based on the severity of harm to a patient resulting from failure to meet that quality attribute. Analytical methods to identify and quantify these PQAs, especially CQAs, are essential for the development of QTPP and implementation of control strategies. Conventionally, a panel of analytical techniques such as size-exclusion chromatography (SEC), ion-exchange chromatography (IEX), hydrophobic-interaction chromatography (HIC), or capillary electrophoresis (CE) is typically used to monitor product quality consistency as well as product variants and impurities at the intact protein level [7-9]. Although these chromatographic and electrophoretic methods widely are used as release assays for biologics [10], they cannot directly monitor biologically relevant PQAs at the molecular level, which does not align with QbD principles. The complexity of biologics attributes and the implementation of QbD strategies demand a multi-attribute method (MAM) that can monitor multiple biologics PQAs at the molecular level in a single assay. Coupling liquid chromatography (LC) to high resolution and high accuracy mass spectrometry (MS) techniques, LC-MS based peptide mapping has become a MAM approach that can identify and quantify multiple attributes.
of biologics simultaneously at the molecular level [6,7,11,12], as well as elucidate the mechanisms associated with degradation [7,13,14]. In a typical MAM workflow, therapeutic protein samples are denatured and reduced to break disulfide bonds between the cysteine residues, which are then alkylated by iodoacetamide. The resulting proteins are digested by a protease, such as trypsin, to generate peptides carrying PQAs. The resulting peptides are separated and analyzed by LC-MS; the peptides are identified using tandem mass spectrometry (MS/MS) and database search software (e.g. Byonic [15], BioPharma Finder, Proteome Discoverer, and MASCOT [16]), and quantified based on the extracted ion chromatography (EIC) of the peptide monoisotopic m/z values using automated software (e.g. Skyline [17] and Pinpoint). The identification and quantification of PQA carrying peptides is used to assess the relative abundance of PQAs in biologics samples. A MAM approach can be applied to in vitro samples such as biologics drug substances, and in vivo samples such as patient sera. In the case of in vivo samples, immunoprecipitation can be first applied to purify therapeutic proteins from patient serum samples before the protein denaturation and digestion steps [18,19]. MS/MS database searches not only help to identify product-related PQAs but also identify product-related impurities, such as sequence variants, as well as process-related impurities, such as HCPs. The database search software and ion peak extraction software enables the semi-automatic data analysis, which dramatically facilitates identification and quantification of these PQAs.

There are three key benefits to the implementation of multi-attribute methods in protein therapeutics development. First, MAM allows for a single assay or for fewer assays to achieve more complete analyses compared to conventional methods. For example, SEC is used for monitoring size-based aggregates or degradants, such as high-molecular weight species (HMW) and low-molecular weight species (LMW); cation exchange chromatography (CEX) is used for characterization of charge-based PQAs, such as C-terminal lysine removal and glycation; Enzyme-Linked Immunosorbent Assay (ELISA) is used to determine the host cell protein levels. A series of these assays are typically required to be developed and utilized to fully characterize therapeutic molecules. In contrast, a single LC-MS based MAM can assess many PQAs simultaneously, including many post-translational modifications (PTMs). Results have shown that data generated using either the LC-MS MAM or conventional analytical methods are comparable [20]. Thus, MAM can decrease the number of assays used during the drug development and potentially reduce costs. More importantly, MAM allows direct monitoring of biologically relevant site-specific PQAs rather than indirect monitoring by conventional methods. For example, CEX chromatography analysis of a monoclonal antibody (mAb) sample usually results in the detection of a limited number of chromatographic peaks (e.g. acidic peaks, main peak, and basic peaks). Each of these broad chromatographic peaks may contain multiple species (e.g. deamidation at multiple amino acid sites). In contrast, MAM can enable quantitation of individual site-specific modifications, such as deamidation in the complementarity-determining regions (CDRs). Since the modifications that occur in CDRs may impact the safety and efficacy of biologics, these modifications are often closely monitored as CQAs. MAM enables direct monitoring CQAs during process development and in vivo circulation to support the risk assessment of the CQAs [18]. Furthermore, applications of MAM during process development and characterization support QbD principles by bridging critical process parameters (CPPs) with drug efficacy information. The correlation between CQAs and drug efficacy can be established by correlating of CQA levels determined by MAM with potency measured by bioassay. Using design of experiment (DoE) approaches, CQAs can be correlated to critical process parameters (CPPs). Thus, drug efficacy can be linked to CPPs to facilitate the process development using QbD principles.

Compared to conventional methods, development of a LC-MS MAM approaches can be more complicated, due to complexity associated with sample preparation, LC separation, MS instrumentation, and data analysis. Each of these steps requires careful consideration of multiple experimental conditions or parameters. Different denaturation and digestion strategies, for example, may be used depending on the types of biologics samples (e.g. IgG1, IgG4, bispecific antibodies, fusion proteins, in vitro samples, or in vivo samples). Denaturation and enzyme digestion conditions, such as pH, digestion time, and denaturants, need to be optimized in order to maximize specific cleavages and minimize modification artifacts induced by sample preparation. LC and column parameters, such as LC gradient, buffer system, column type and column temperature, need to be selected for optimal separation of peptides. Mass spectrometry parameters, such as tuning method and acquisition method, must be optimized. Data analysis parameters, such as search parameters and ion peak extraction parameters, should also be considered. LC-MS based biologics characterization methods are typically classified into three approaches: the top-down approach (i.e. the gas phase fragments of intact biomolecules), the middle-up approach (i.e. the gas
phase fragmentation analysis of mAb fragments yielded by reduction or limited proteolysis, and the bottom-up approach (i.e. peptide mapping). The bottom-up approach is generally used in LC-MS based MAM methods because it can provide site-specific PTM and sequence variants information. On the other hand, the top-down approach can be used to analyze the degradation products and impurities, such as characterization of HMW and LMW species using native-SEC-MS [21]. Depending on the MS analysis approaches, the separation strategy and MS acquisition method should be carefully chosen and optimized.

Because of the above mentioned parameters, the development of a LC-MS MAM could be time-consuming. Using a DoE-based approach can therefore greatly facilitate LC-MS MAM development. The purpose of DoE is to establish a cause-and-effect relationship between the dependent variables (responses) and independent variables (factors) by testing a series of factor values (levels). For example, the protease digestion step aims to establish the correlation between digestion results (e.g. maximizing the sequence coverage while minimizing modification artifacts, such as oxidation and deamidation) and digestion factors (e.g. denaturant concentration, alkylation reagent concentration, pH, digestion time, and protein/enzyme ratio). To this end, a set of experiments with varying levels of experimental factors are performed to establish the correlation between responses and factors. Then, the proper experimental parameters can be determined based on the established correlation.

The LC-MS MAM approach can be applied to all stages of a therapeutic protein development, including candidate selection, process development, stability and comparability assessment, quality control, and drug disposition. For example, LC-MS MAM has been used for characterization and relative quantification of amino acid modifications and glycoform distribution of monoclonal antibodies [20]. LC-MS MAM can also be used to monitor lysine glycation of mAb in bioreactors. Lysine glycation is a non-enzymatic modification that can potentially affect conformation, efficacy, and immunogenicity [22]. The level of glycation at a particular site depends on primary sequence and higher order structures, bioreactor glucose concentration and feed strategy, pH and temperature of buffer streams, and hold times of in-process intermediates [22]. The LC-MAM analysis allows for simultaneous monitoring of all site-specific lysine glycation within a protein expressed in a bioreactor over time, which provides timely information to adjust feed strategy during process development. In addition, the LC-MS MAM approach can be used to monitor potential changes to the PTM profile of a given therapeutic mAb in vivo [18]. Monitoring of PTMs within biologics in vivo has been recently recommended by FDA [23]. The potential susceptibility of a therapeutic protein to modifications in vivo may result in loss of efficacy or induction of immune responses. Obtaining this information early in product design, candidate selection, and drug development may facilitate product engineering to enhance the stability of the product in the in vivo milieu. Using LC-MS MAM, site-specific chemical and enzymatic modifications, such as deamidation, oxidation, N-terminal pyroglutamate formation, C-terminal lysine removal, and glycosylation profiles can be simultaneously quantified over time following drug administration. These quantitative results can be further used to build up mathematical models to assess patient exposure to PQAs, which helps to estimate the impact of process changes on product attribute exposure [18,24].

The high complexity of biologics supports the use of a MAM-based assay to analyze multiple PQAs of biologics in a single assay. Compared to conventional methodologies, the LC-MS MAM approach provides direct identification and quantification of the biologically relevant PQAs, and embraces QbD principles through establishing the correlation between CQAs and CPPs to achieve increased efficiencies in manufacturing and to meet higher regulatory expectations. The application of MAM assays provides quantitative information to support continuous drug development from early stage drug candidate selection and process development to pre-clinical and clinical studies. With the advancement of mass spectrometry technology and instrument automation, fully automated LC-MS MAM platform including data analysis could be developed in the near future that provides much more in-depth quantitative information of PQAs than current conventional analytical methods to support the biologics development.

References


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