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EXPERT OPINION

LC-MS multi-attribute method for characterization of biologics

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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) presenting 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

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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 al-

lows 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

1. U.S. Department of Health and Human Services & Food and Drug Administration. Guidance for Industry, Q6B Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products (1999). Available at http://www.fda.gov/downloads/drugs/guidancecomplianceregulatory-information/guidances/ucm073488.pdf

2. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Pharmaceutical Development (Q8) (2009). Available at http://www.ich.org/products/guidelines/quality/article/quali-

ty-guidelines.html.

3. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Quality Risk Management Q9 (2006). Available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.

4. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Pharmaceutical Quality System (Q10) (2008). Available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.

5. International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use. Development and Manufacture of Drug Substances (Q11) (2012). Available at http://www.ich.org/products/guidelines/quality/article/quality-guidelines.html.

6. Beck A, Wagner-Rousset E, Ayoub D, Van Dorsselaer A, Sanglier-Cianferani S. Characterization of therapeutic antibodies and related products. Anal Chem 85, 715-736 (2013).

7. Sandra K, Vandenheede I, Sandra P. Modern chromatographic and mass spectrometric techniques for protein biopharmaceutical characterization. J Chromatogr A 1335, 81-103 (2014).

8. Valliere-Douglass J, Wallace A, Balland A. Separation of populations of antibody variants by fine tuning of hydrophobic-interaction chromatography operating conditions. J Chromatogr A 1214, 81-89 (2008).

9. Zhao SS, Chen DD. Applications of capillary electrophoresis in characterizing recombinant protein therapeutics. Electrophoresis 35, 96-108 (2014).

10. Federici M, Lubiniecki A, Manikwar P, Volkin DB. Analytical lessons learned from selected therapeutic protein drug comparability studies. Biologicals 41, 131-147 (2013).

11. Kaltashov IA, Bobst CE, Abzalimov RR, Wang G, Baykal B, Wang S. Advances and challenges in analytical characterization of biotechnology products: mass spectrometry-based approaches to study properties and behavior of protein therapeutics. Biotechnol Adv 30, 210-222 (2012).

12. Zhang Z. Large-scale identification and quantification of covalent modifications in therapeutic proteins. Anal Chemistry 81, 8354-8364 (2009).

13. Goetze AM, Liu YD, Zhang Z, Shah B, Lee E, Bondarenko PV, et al. High-mannose glycans on the Fc region of therapeutic IgG antibodies increase se-

rum clearance in humans. Glycobiology 21, 949-959 (2011).

14. Shah B, Jiang XG, Chen L, Zhang Z. LC-MS/MS peptide mapping with automated data processing for routine profiling of N-glycans in immunoglobulins. J Am Soc Mass Spectrom 25, 999-1011 (2014).

15. Bern M, Kil YJ, Becker C. Byonic: advanced peptide and protein identification software. Curr Protoc Bioinformatics (2012) https://doi.org/10.1002/0471250953.bi1320s40

16. Perkins DN, Pappin DJ, Creasy DM, Cottrell JS. Probability-based protein identification by searching sequence databases using mass spectrometry data. Electrophoresis 20, 3551-3567 (1999).

17. MacLean B, Tomazela DM, Shulman N, Chambers M, Finney GL, Frewen B, et al. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. Bioinformatics 26, 966-968 (2010).

18. Xu X. *In vivo* characterization of therapeutic monoclonal antibodies. J Appl Bioanal 2(1), 10-15(2016).

19. Yin S, Pastuskovas CV, Khawli LA, Stults JT. Characterization of therapeutic monoclonal antibodies reveals differences between *in vitro* and *in vivo* time-course studies. Pharm Res 30, 167-178 (2013).

20. Rogers RS, Nightlinger NS, Livingston B, Campbell P, Bailey R, Balland A. Development of a quantitative mass spectrometry multi-attribute method for characterization, quality control testing and disposition of biologics. mAbs 7, 881-890 (2015).

21. Haberger M, Leiss M, Heidenreich AK, Pester O, Hafenmair G, Hook M, et al. Rapid characterization of biotherapeutic proteins by size-exclusion chromatography coupled to native mass spectrometry. mAbs 8, 331-339 (2016).

22. Miller AK, Hambly DM, Kerwin BA, Treuheit MJ, Gadgil HS. Characterization of site-specific glycation during process development of a human therapeutic monoclonal antibody. J Pharm Sci 100, 2543-2550 (2011).

23. U.S. Department of Health and Human Services & Food and Drug Adminstration. Guidance for Industry, Immunogenicity Assessment for Therapeutic Protein Products. (2014). Available at http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm338856.pdf

24. Goetze AM, Schenauer MR, Flynn GC. Assessing monoclonal antibody product quality attribute criticality through clinical studies. mAbs 2, 500-507 (2010).

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