Analytical Solutions from Cell Culture to Biologics Development and Quality Control

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Overview

- Biopharmaceuticals such as monoclonal antibodies (mAbs) and antibody drug conjugates (ADCs) have emerged as revolutionary therapeutic agents for an array of human diseases. Accurate characterization of these molecules is vital to the development of biotherapeutics.
- It is essential to know the properties of biotherapeutic products to optimize bioprocess production from cell culture, product formulation, and dose.
- Shimadzu's innovative instrumentation, like LC, LC/MS, and MALDI, with industry-leading LabSolutions software were used to characterize cell culture

1. Introduction

In this study, a multi-instrument platform was used to observe the process of cell culture production to the development of biologics. A CHO cell line was chosen for the study of culture solutions that were sampled every 24 hours and supernatants obtained by centrifugation. An automatic pretreatment with the Shimadzu C2MAP-2030 system was used for removing proteins from culture supernatants by precipitation with organic solvent, suction filtration, and automated transfer of samples for LC-MS/MS analysis (TripleQuad mass spectrometry). In a 20-minute method cycle, the system analyzed 125 metabolites with a wide range of chemical properties simultaneously using high-speed scanning and positive/negative ionization switching. During cell culture, an atomic absorption spectrometry (AAS) system was used to quantify element concentrations based on the absorption of specific light wavelengths (data not shown). The high concentrations of Mg and Zn were measured by flame AA and the trace elements (Cu, Mn, Co, and Fe) were determined by graphite furnace AA (GFAA). Finally, mAbs and ADCs are analyzed using LC, LC-MS and MALDI-MS.

2. Methods

A variety of instruments have been used for analysis, including UHPLC, LCMS triple quad, LCMS QTOF, and MALDI. A brief summary of the methods is given below:

- **LC Method**

Analyzing biomolecules on a wetted surface of an UHPLC instrument presents some critical challenges. Nexera XS inert is extremely resistant to mobile phases containing high salt concentrations. A combination of the high-pressure tolerance of a UHPLC system with an inert flow path that had no wetted metal surfaces in the sample flow path ensured ultra-high corrosion resistance. This study utilized different types TSKgel columns. Different modes of analysis were performed, including IEX for charge valiant analysis and HIC for DAR characterization and SEC for ADC analysis and mAb optimization.

- **LCMS-9030 QTOF Method**

Shimadzu Q-TOF LCMS-9030 was used in positive mode for both MS and MS/MS scans using Restek Ultra C4 column for the intact and peptide mapping. The analysis and characterization of mAbs was performed with Protein Metrics software. The workflows were customized for all types of analyses, including intact protein, subunits, peptide fragments, and deglycosylation analyses.

- **LCMS-8060 (Triple Quad) Method**

Shimadzu LCMS-8060 was operated in MRM mode for targeted quantitation of peptide fragments containing β-amyloid antibody (6E10) CDR's (complementarity determining regions). This study utilized Skyline targeted proteomics software (MacCoss et al., University of Washington, Seattle, Washington) to predict precursors, product ions, collision energies, and retention times.

- **MALDI Method**

The benchtop linear MALDI-8020 MS was used for determining the degree of small-molecule modification biomarker analysis for the cancerous cell line to mimic the cell culture monitoring. This technique was also applied in monitoring cell culture, mAb characterization and N-glycan analysis. MALDI-8030 is the latest benchtop MALDI Mini, which can run both positive and negative modes. eMSTAT Solution software was used to analyze the data in order to find biomarkers. The sample was prepared by mixing with matrix and spotted directly to the plate and dried prior to being analyzed by 200 Hz solid state laser.

- **Data processing**

Chromatographic data were collected and analyzed by LabSolutions software. LC-MS data were taken by LabSolutions and analyzed by Protein Metrics and Skyline software. The MALDI-MS data were collected by MALDI Solutions and analyzed with eMSTAT Solution and Protein Metrics software.

2. Results

Quantitation of Metal Elements and Metabolites in Culture by AA and C2MAP

Figure 1: Analysis Process Flow with C2MAP System.

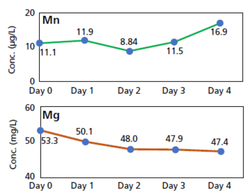

Figure 2: Time course of Mn and Mg concentration in culture supernatant

Cell Culture Solution Monitoring

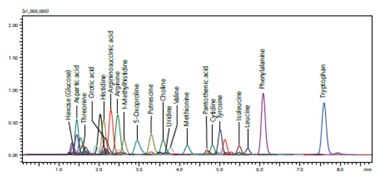

Figure 3: Transitions in symmetry factor of ATP by the number of injections.

The C2MAP system can automate the deproteinization process for up to 65 culture supernatant samples. By linking it to an LC-MS system, the entire process from pretreatment to LC/MS/MS measurement can be executed seamlessly.

Extracellular Vesicle Analysis by MALDI-8020

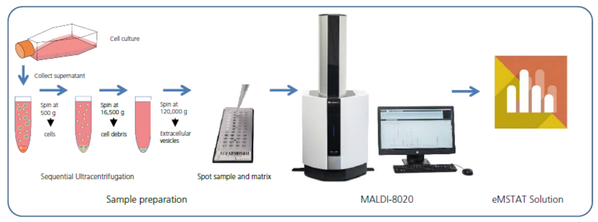

Figure 4: Workflow for Extracellular Vesicle Analysis

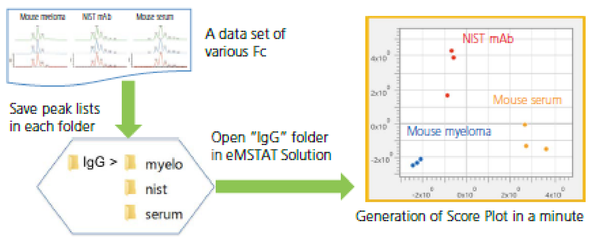

Figure 5: Classification of Fc Region Using eMSTAT Solution

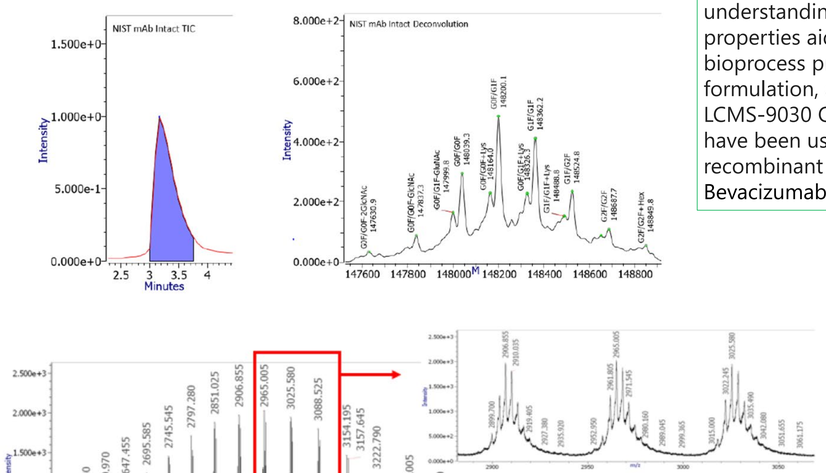

Figure 6: NIST mAb Intact TIC chromatogram (top, left), deconvoluted spectrum (top right), and mass spectrum (bottom).

Figures 1-3 depict the cell culture media analysis procedure and results using C2MAP. C2MAP analysis of cell culture supernatants was carried out for a simultaneous multi-component monitoring. As a result, kynurenine and 2-aminoadipic acid were identified as biomarkers that indicated distinctive changes in undifferentiated iPS cells and ectodermal differentiated cells. The study showed that the identified biomarkers were closely related to the undifferentiated state of cells and the differentiated state of ectodermal cells. Using the C2MAP system, these results demonstrated that it is possible to evaluate the state of cells non-invasively. Figures 4-5 show the MALDI-MS analysis of cell extracellular vesicles from cancerous cell lines. Chemical modifications mAb and ADC can also be analyzed with this method.

Figure 7: Deconvoluted mass spectrum of NIST mAb.

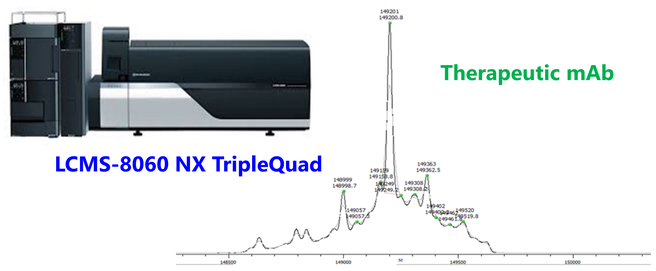

Figure 8: Deconvoluted mass spectrum of Bevacizumab.

Accurate characterization of monoclonal antibodies is essential to development of biotherapeutics. Thorough understanding of biotherapeutic properties aids in the optimization of bioprocess production, product formulation, and product dosage. LCMS-9030 QTOF and Nexera XS inert have been used to characterize the recombinant human NIST mAb and Bevacizumab

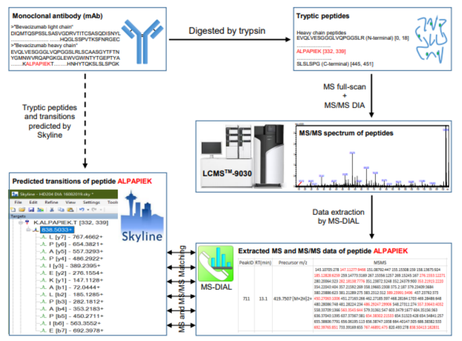

Figure 9: The *de novo* peptide sequencing approach on LCMS-9030 (QTOF) for characterization of mAb. The peptide ALPAPIEK was used as an example for elastration.

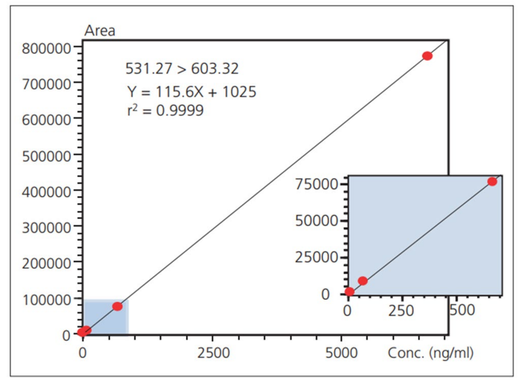

Figure 10: Calibration Data for the Transition 531.27 > 603.32 on a Linear Scaling. Detection of trypsin fragments containing CDR1-3 using standard monoclonal antibodies subjected to trypsin digestion (Data Analysis by Skyline and obtained by LabSolutions and Triple Quad instrument).

N-Linked Glycan Analysis

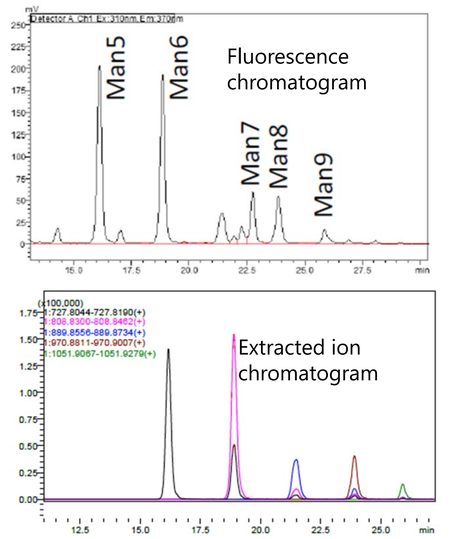

Figure 11: Fluorescence chromatogram (upper) and extracted ion chromatogram (lower) for procainamide labeled RNaseB Glycans .

Drug-to-Antibody Ratio by Nexera XS inert

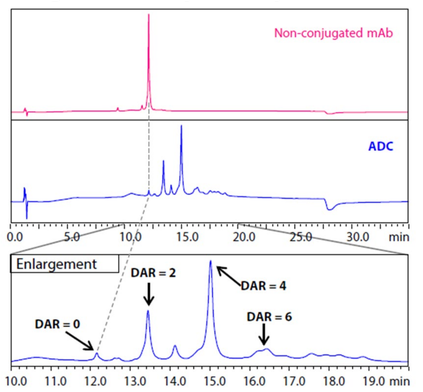

Figure 12: Result of drug-to-antibody ratio (DAR) analysis.

The application of MS/MS DIA on the Shimadzu LCMS-9030 (Q-TOF) mass spectrometer was demonstrated for de novo sequencing of mAb peptides based on in-depth peptide mapping. Since intact proteins are complex, there are several ways to characterize fully a mAb. An antigen's complementary determining region (CDR) that is directly in contact with its surface. Both inter- and intra-chain disulfide bonds link two identical heavy chains (HC) and two identical light chains (LC). The disulfide bonds can be reduced to allow the HC and LC to be analyzed independently. Protein fragments are cleaved into peptides by enzyme digestion with trypsin, Glu-C, or Lys-C. By using specialized software, the resulting masses can be compared to a theoretical sequence to confirm the sequence in silico. During post translational modification (PTM), oligosaccharides are covalently attached to the protein on the HC subunit. An N-glyosidic bond is the most common glycosylation site in human mAbs. In addition to their regulatory and recognition functions, these N-glycans can be cleaved by bacterial enzymes. Figures 6-11 illustrate the complete analysis of mAb such as intact proteins, MRM based peptide quantification, de novo peptide sequencing, and N-glycan monitoring using LCMS-9030, LCMS-8060/8050 (TripleQuad), LabSolutions, Protein Metrics and Skyline software. Additionally, NIST mAb and therapeutic mAb were characterized in order to identify them effectively. Figure 12 illustrates the Bioinert UHPLC analysis of mAb and ADC. The ADC separation was achieved with SEC by suppressing the interaction between the ADC and stationary phase. IEX separated acidic and basic charge variants according to the charge strength. Several small-molecule drugs linked to the antibody were used to separate a cysteine-linked ADC with HIC. Nexera XS inert's excellent corrosion resistance ensured stable data for antibody drug analysis or any type of analysis requiring a mobile phase containing corrosive salts.

4. Conclusions

- Full characterization of product monoclonal antibodies (mAbs) was undertaken by LCMS, MALDI and UHPLC.
- Peptide mapping, peptide sequencing, glycosylation, and oxidation of target mAbs analyzed by QTOF, triple quadrupole, and MALDI mass spectrometry was performed to characterize and control biologics production.
- Additionally, n-glycans were analyzed using UHPLC with fluorescence detection after labeling with pyridylamino (PA)-glycan and 2-aminobenzamide (2-AB)- glycan.