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Antibody N-glycopeptide Analysis

Introduction

Monoclonal antibodies (mAbs) are widely used in pharmaceutical research, primarily as therapeutic agents. Post-translational modifications (PTMs) are critical quality attributes in mAb production, influencing the mAbs stability, efficacy, and function. Among these modifications, protein glycosylation—the attachment of sugar units—plays a key role in determining the biological properties of mAbs. In particular, Fc glycosylation significantly impacts effector functions within the immune system, affecting antibody-dependent cellular responses and overall therapeutic performance.

Why use N-glycopeptide Analysis?

In one type of protein glycosylation complex, sugar units are attached to specific asparagine amino acid residues in a protein (N-glycans). N-glycopeptide analysis can provide site specific information on the glycosylation profile of N-linked glycans and is particularly useful in proteins with multiple N-glycosylation sites. The alternative N-glycan release method—using PNGase F—is also useful but can only provide an average of the glycan species present across multiple sites.

Methods in Brief

Samples are first denatured and treated with a reducing agent such as dithiothreitol (DTT). DTT breaks disulfide bonds formed between two cysteine residues within a protein which makes the protein chain more accessible to protein digestion. This is followed by alkylation of the protein's free thiols, which prevents reformation of the disulfide bonds. The mixture is then subjected to digestion of the protein backbone with one or more proteases to create smaller peptide fragments. The sugar units on the asparagine residues are not affected by these steps and thus remain on the peptide, encompassing the amino acid sequence around the N-glycosylation site. These peptide fragments are then analyzed by Liquid Chromatography Mass Spectroscopy (LCMS) which accurately measures the masses of each peptide. Within the LCMS instrument, the peptides can be fragmented into smaller pieces from which additional information can be obtained about their composition and order of amino acid linkage (MS/MS or MS2). This includes fragments unique to glycans.

Chromatography was preformed using a Dionex™ UltiMate™ 3000 HPLC in tandem with a Thermo Q Exactive™ Hybrid Quadrupole-Orbitrap™

- Stationary Phase:
  Waters Acquity HSS T3 1.8 µm, 1.0x100 mm column
- Mobile Phase A:
  Water, 0.1% Formic Acid
- Mobile Phase B:
  Acetonitrile, 0.1% Formic Acid

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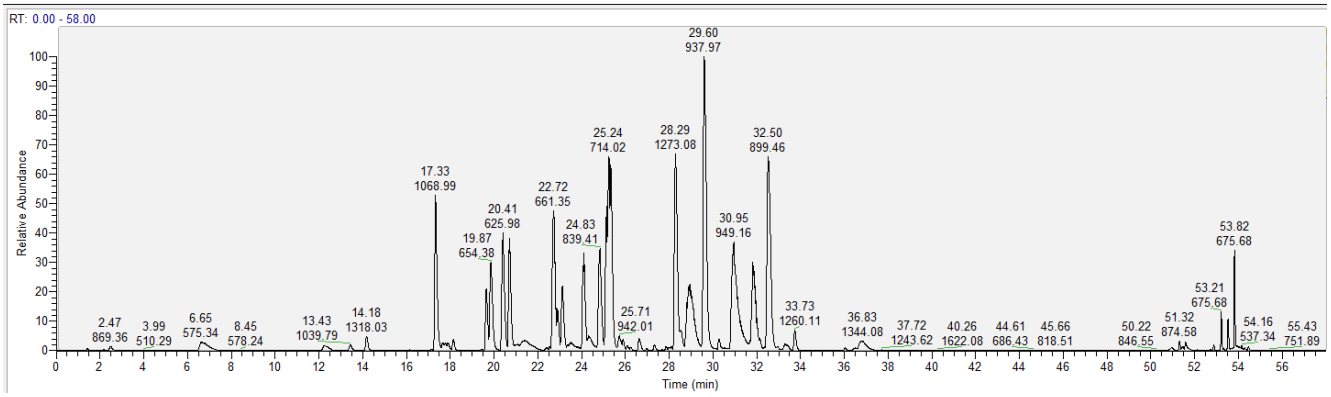
Proof of Concept

Bevacizumab is a recombinant mAb medication used to treat certain types of cancer. It works by blocking the growth of new blood vessels (angiogenesis), which tumors need to survive and spread. Below is the LCMS base peak chromatogram for bevacizumab.



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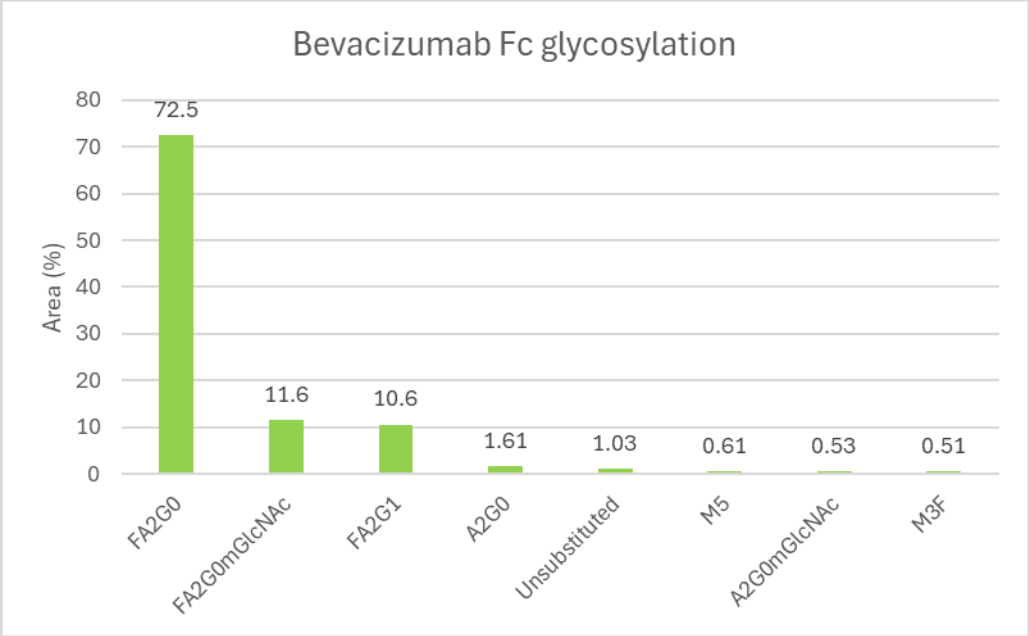
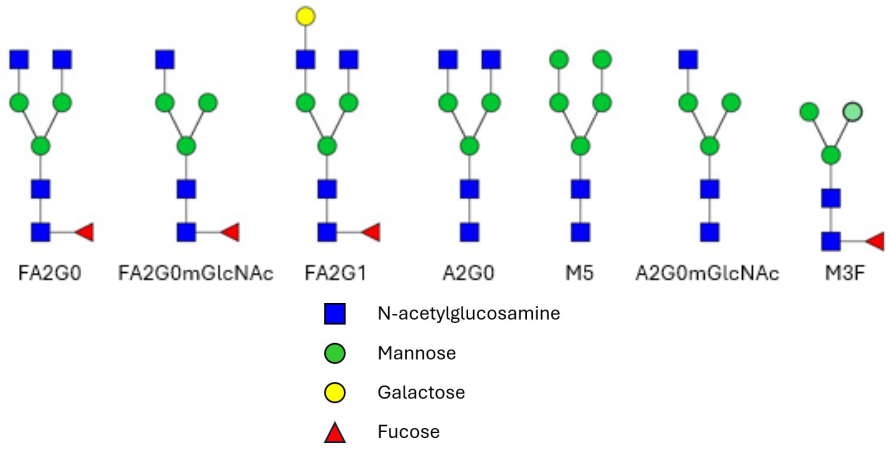
Bevacizumab has a single site of N-glycosylation in the Fc region of the antibody. Sixteen different glycan species could be detected by glycopeptide LCMS. Below is a bar graph showing the relative percentages of the most abundant species observed (plotting only those with >0.4% abundance). This sample of Bevacizumab lacks virtually any sialylation and is predominantly fucosylated.



Representative structures corresponding to the glycans in the bar graph



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Why Spectrus is the right choice for N-glycopeptide analysis

Spectrus' combination of experience in sample preparation together with state-of-the-art mass spectrometry instrumentation allows Spectrus to provide the highest degree of confidence in analysis of N-glycosylation PTMs in your biomolecules.

Literature

Mayra G. Quiñonez-Alvarado, Paulina Chávez-Hurtado, Jesús C. Caro-Palomera, Oriana L. Niño-Trejo, José I. Jiménez-Dolores, Patricia Muñoz-Villegas, Leopoldo Baiza-Durán, Juan D. Quintana-Hau Glycosylation differences of an anti-VEGF monoclonal antibody (PRO-169) and its extensive comparison with Bevacizumab, *Biologicals* **2023**, *84*, 101711