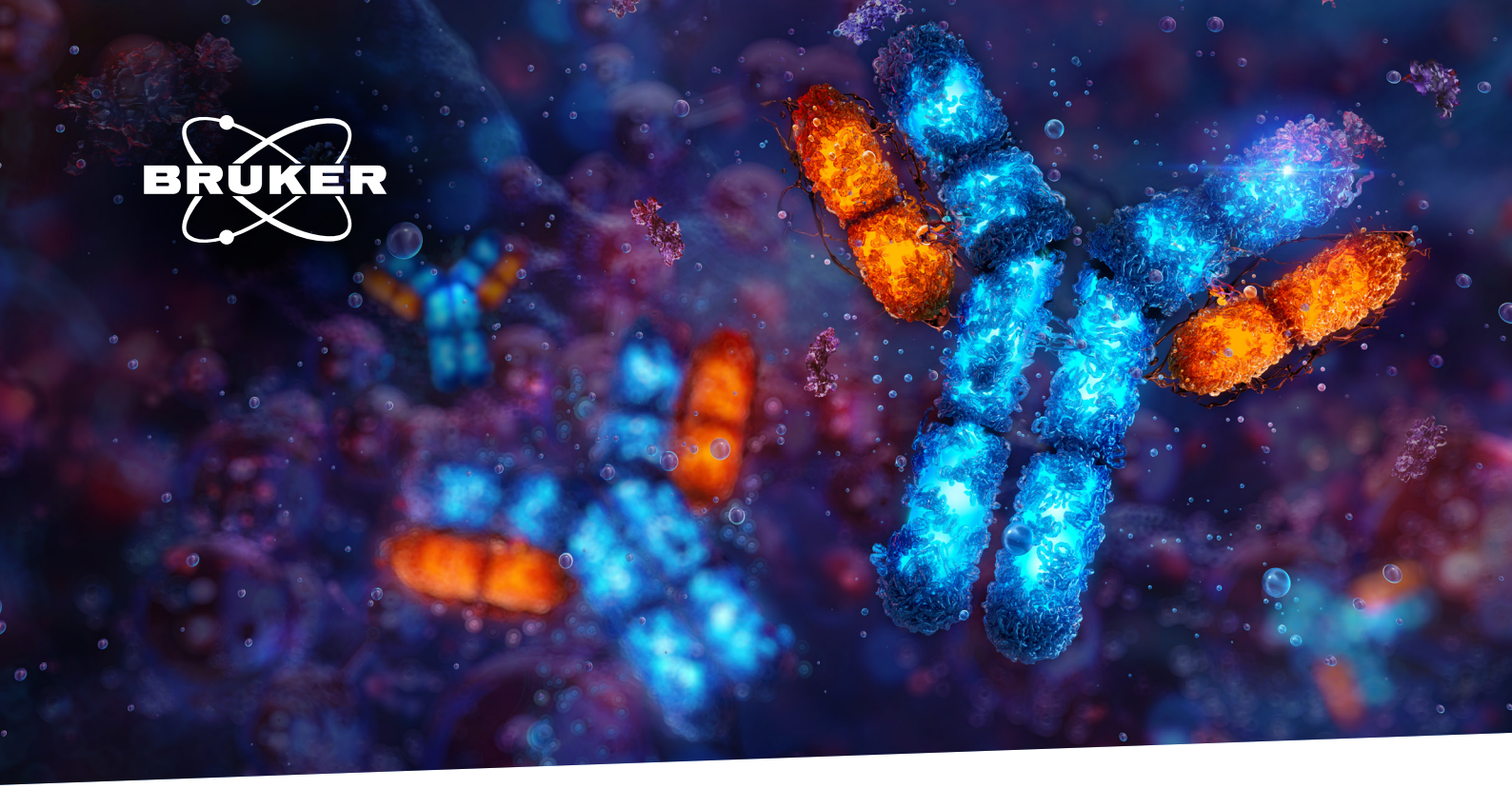

N-glycan profiling of biotherapeutic monoclonal antibodies using Bruker's timsTOF fleX and neofleX platforms and BioPharma Compass Software: a novel tool for smart lead development and selection in pharma-biotech R&D.

Adding glycosylation to the repertoire of high-troughput drug candidate screening.

Abstract

- A MALDI-MS-based workflow with automated liquid handling for the rapid, high-throughput profiling of N-glycan microheterogeneity present in therapeutic antibody and other therapeutic protein candidate molecules
- Fully compatible with laboratory automation platforms and liquid handlers through the use of Bruker's 384-well-format AnchorChip MALDI targets
- Bruker's BioPharma Compass software facilitates rapid MS1 spectral annotation and N-glycan identification through its integrated glycan search platform.
- As a exemplary proof-of-principle, a number of nivolumab biosimilars both the same source, and from different manufacturers were analyzed, yielding differential N-glycan profiles.

Keywords:
Glycobiology, antibody glycosylation, glycomics, N-glycans, pharma, biopharma, timsTOF fleX, neofleX, BioPharma Compass, lead-development; lead-selection, lead-optimization; lead-screening

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Introduction

Therapeutic antibodies have shown great promise for the treatment of hematologic, autoimmune, cardiovascular diseases and cancer, among other conditions [1]. While antibody-antigen binding is primarily driven by amino acids in the complementarity-determining regions (CDRs), antibody glycosylation can mediate antigen recognition, humoral response, and anti-drug antibody (ADA) response [2, 3]. Likewise, glycosylation profiles may affect drug safety, such as immunogenicity and off-target effects, as well as pharmacokinetic and pharmacodynamic parameters, such as half-life, clearance, solubility, stability, and aggregation. Antibodies of the IgG class contain conserved sites of N-glycosylation at each Asn^{297} residue in the CH2 regions of their Fc domains [4]. Monitoring the N-glycans present at these sites is an important quality metric, as mammalian host cell lines used to produce therapeutic antibodies have the potential to generate immunogenic responses due to the integration of non-human glycosylation patterns [3]. Additionally, alterations in therapeutic antibody glycosylation can affect therapeutic antibody internalization and degradation rates, as well as T cell recognition [3]. Such alterations can arise from variations in cell culture conditions which relate to substrate availability, as well as pH and temperature [5].

Here we present a MALDI-MS-based method for rapid and efficient profiling N-glycans present on therapeutic antibodies and proteins amenable to drug molecule screening applications. To exemplify this, we carried out a study in which N-glycans were enzymatically cleaved from a number of nivolumab biosimilars using PNGase F, and their relative abundances were profiled via an LC-free mass-spectrometry approach. Such an approach is also readily extensible to additional, complimentary glycopeptide analysis through the substitution of an endopeptidase for PNGase F, where site specificity is of interest. The LC-free workflow presented in this work allows therapeutic antibodies to be successfully differentiated on the basis of their N-glycan profiles. The workflow is compatible with lab automation platforms for monitoring N-glycosylation in the context of very high-throughput requirements of upstream therapeutic antibody lead development.

Experimental

Sample preparation

Therapeutic antibody samples were resuspended in LCMS-quality water and desalted in Amicon centrifugal filters (10 kDa MWCO). N-linked glycans were enzymatically cleaved by PNGase F at 37°C and the released N-glycans were enriched using a hydrophilic interaction SPE plate coupled with a vacuum manifold system. Samples were dried down, resuspended in water, and spotted on to a Bruker MTP SmallAnchor 384 MALDI target using SDHB (10 g/L in 50:50 ACN:H_2O,v/v; 5 mM NaCl) as the chemical matrix.

MALDI-MS Analysis

All MS data was acquired on a Bruker timsTOF fleX operated in MALDI mode or a Bruker NeofleX benchtop MALDI-TOF/TOF instrument. Profile spectra were acquired in positive reflector mode using 12,000 laser pulses applied across a 220 μm x 150 μm target, with an m/z detection range set to 1000 – 3000 Th.

External calibration was performed on the timsTOF fleX using Agilent's ESI-L Low Concentration Tuning Mix (G1969-85000) and on the NeofleX using an equimolar mixture of G0, G0F, G2, and G2F glycans purchased from NIST (SRM 3655).

Data Analysis

MS spectra were imported into BioPharma Compass (BPC) 2025 and BPC's glycan search function was used (Carbbank database) to assign N-glycan compositions to MS^1 peaks. Normalized intensity and normalized intensity log ratios were calculated in the R programming environment and plotted using the ggplot2 package.

Results

N-glycan profiling

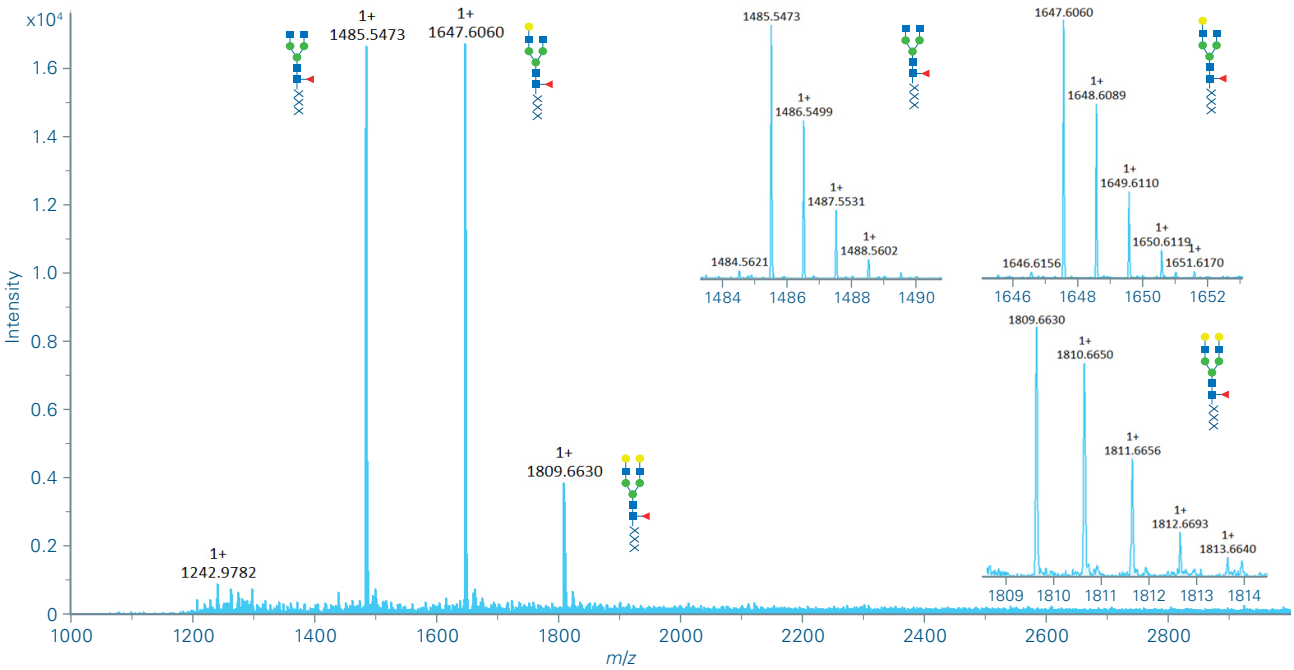

Figure 1. **(+)MALDI-TOF spectrum of PNGase F released N-glycans from SigmaMAb nivolumab monoclonal antibody mass spectrometry standard.**
N-glycan structures assigned in the spectrum are in accordance with [6]. Three fucosylated N-glycans (G0F, G1F, and G2F) are among the top 3 most abundant glycans detected for each biosimilar (Figure 2).

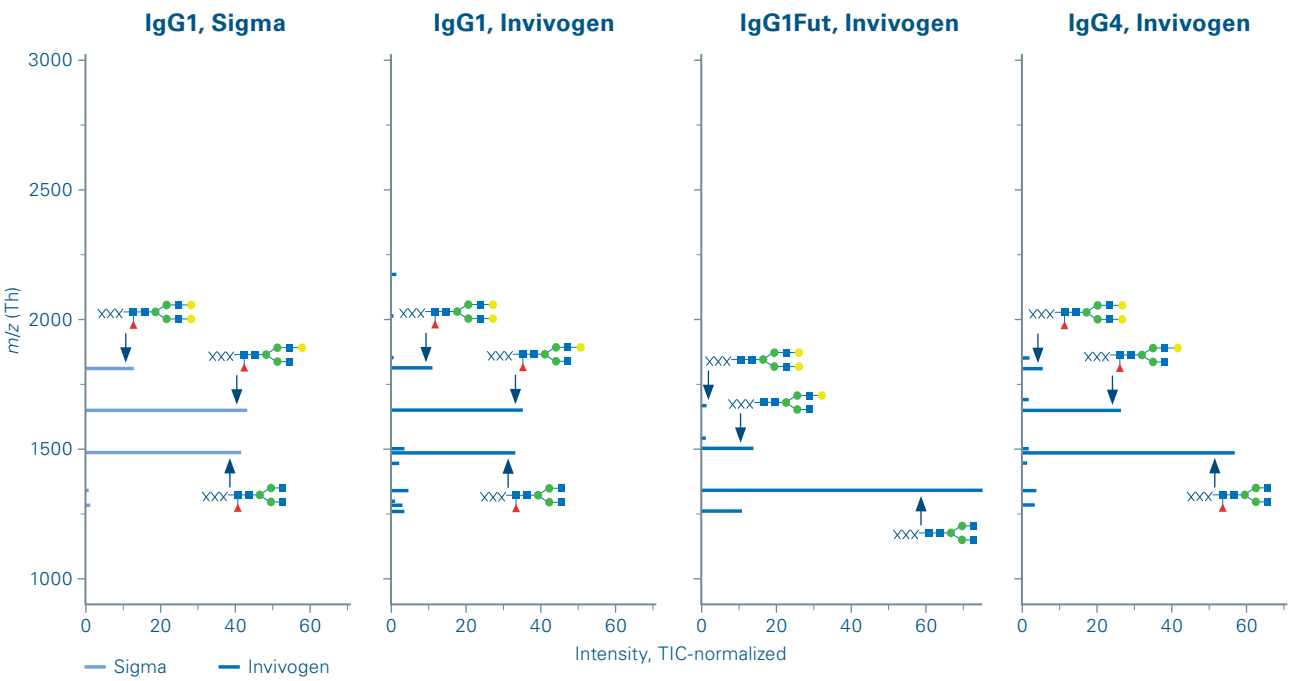

Figure 2. **TIC-normalized N-glycans from MALDI-MS analysis of Nivolumab biosimilars.**
'IgG1Fut' denotes a biosimilar that has been engineered not to produce fucosylated N-glycans.

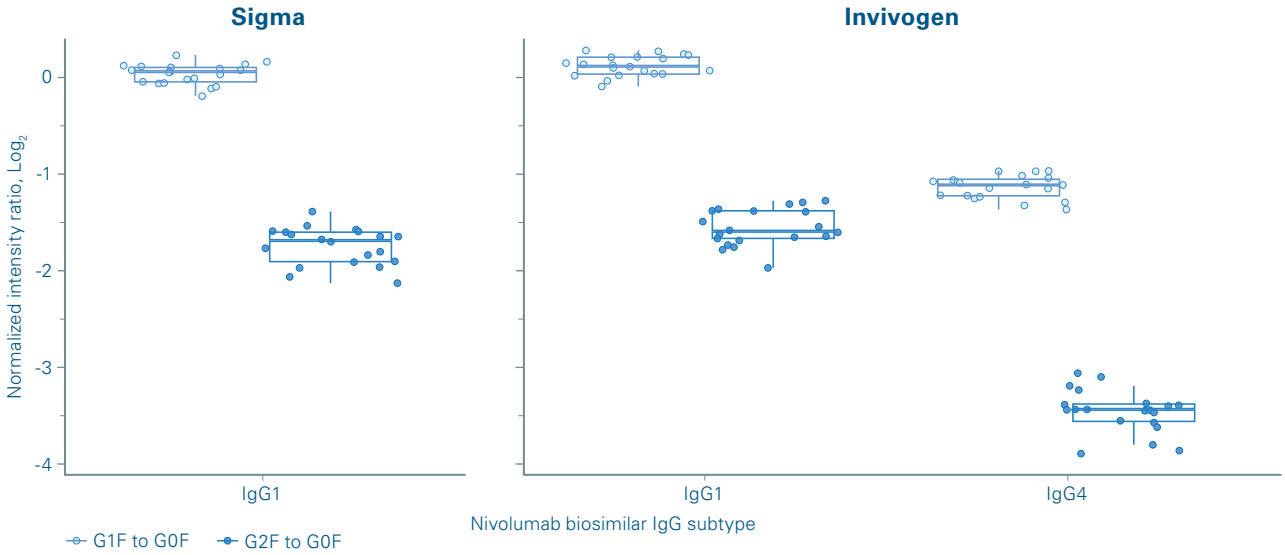

Figure 3. Ratios of TIC-normalized intensities for the top three most abundant N-glycans across Nivolumab biosimilar products featuring fucosylated N-glycans.
The log_2-transformed TIC-normalized intensity ratios (G1F:G0F and G2F:G0F) are shown for each of three nivolumab biosimilars. The biosimilars featuring IgG1-subtype Fc regions show similar G1F:G0F and G2F:G0F intensity ratios (mean values of 1.02 and 1.08 for Sigma and Invivogen's G1F:G0F ratios, respectively; mean values of 0.30 and 0.34 for SIgma and Invivogen's G2F:G0F ratios, respectively). The Invivogen nivolumab biosimilar featuring an IgG4-subtype Fc region showed distinct G1F:G0F and G2F:G0F intensity ratios relative to either of the biosimilars bearing IgG1-subtype Fc regions and fucosylated N-glycans: mean values of 0.46 and 0.09, respectively.

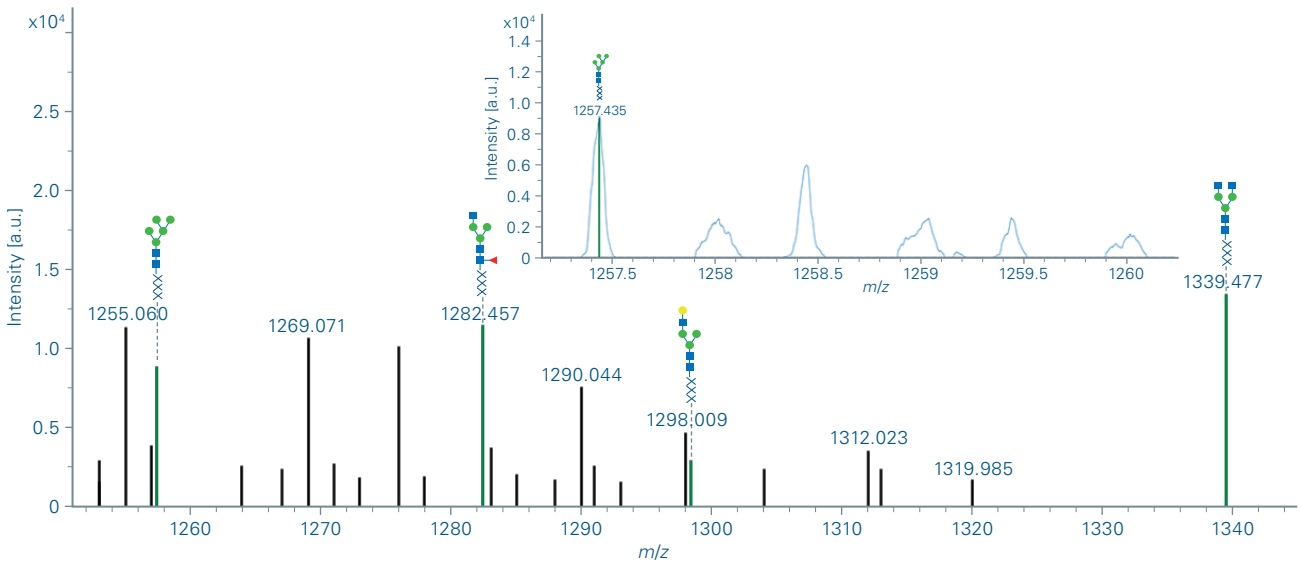

Figure 4. (+)MALDI-TOF spectrum of PNGase F released N-glycans from Invivogen nivolumab biosimilar with IgG1-subtype Fc region.
N-glycan structures assigned in the spectrum are in accordance with [6]. Invivogen's nivolumab biosimilar (IgG1-subtype Fc region) features a Man5 N-glycan detected at 1257.422 (Th).

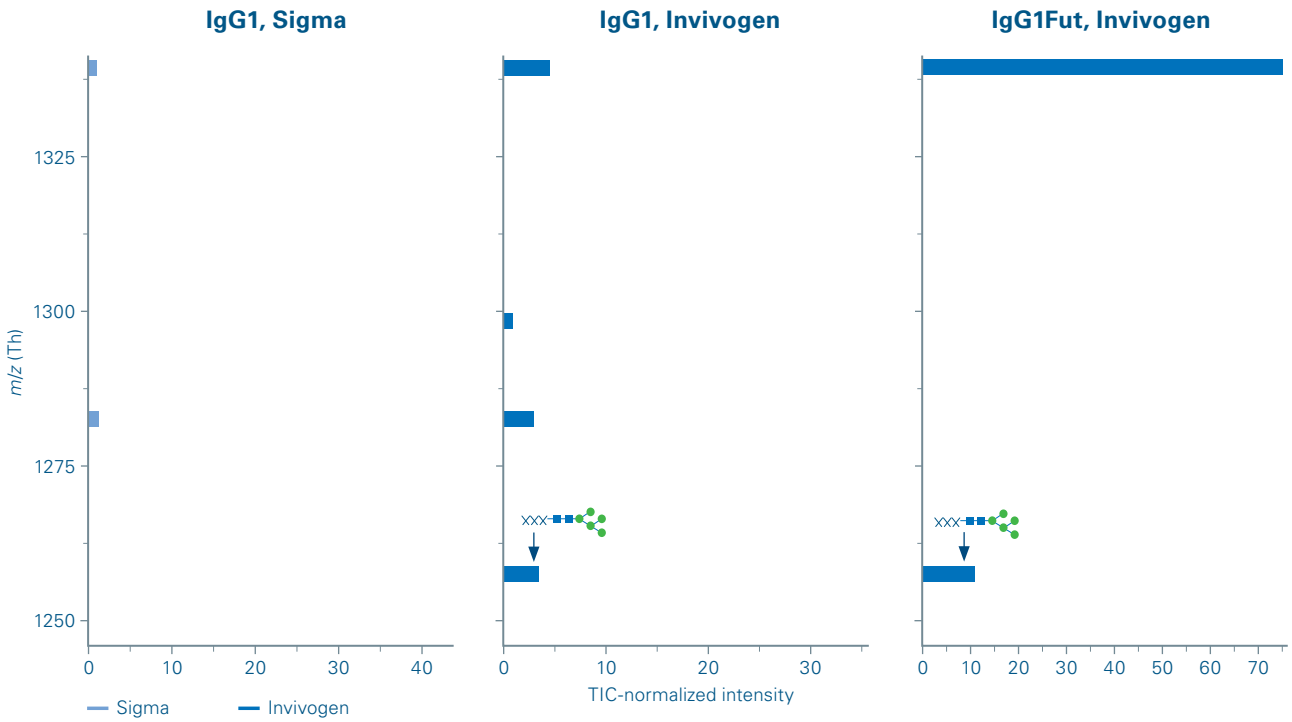

Figure 5. TIC-normalized intensities for N-glycans detected in the *m/z* range 1250 – 1350 (Th) across nivolumab biosimilar products featuring IgG1 Fc Regions.

Conclusions

- GlycoTyper Pharma is a novel, dedicated application platform to allow rapid and sensitive glycoprofile characterization of biotherapeutic candidate molecules in screening-scale high throughput mode.
- Proof-of principle for this is presented using a range of nivolumab biosimilar molecules that the platform demonstrated to differ with regard to their G1F:G0F and G2F:G0F N-glycan ratios.
- Nivolumab biosimilars produced by individual suppliers differed from each other with regard to presence of a Man5 N-glycan at *m/z* 1257.422 in the MS1 spectrum.
- Coupling the timsTOF flex and neofleX with BioPharma Compass (BPC) software enables straightforward annotation of MS^1 spectra using BPC's glycan search platform.
- The workflow illustrated here is fully compatible with lab automation platforms for integration in a therapeutic antibody development and manufacturing quality management system.

References

[1] Sharma P, Joshi RV, Pritchard R, Xu K, Eicher MA (2023). *Therapeutic Antibodies in Medicine*. Molecules. **28**(18):6438. doi: 10.3390/molecules28186438. PMID: 37764213; PMCID: PMC10535987.

[2] Lodge J, Kajtar L, Duxbury R, Hall D, Burley GA, Cordy J, Yates JWT, Rattray Z (2025). *Quantifying antibody binding: techniques and therapeutic implications*. MAbs. **17**(1):2459795. doi: 10.1080/19420862.2025.2459795. Epub 2025 Feb 16. PMID: 39957177; PMCID: PMC11834528.

[3] Wolf B, Piksa M, Beley I, Patoux A, Besson T, Cordier V, Voedisch B, Schindler P, Stöllner D, Perrot L, von Gunten S, Brees D, Kammüller M (2022). *Therapeutic antibody glycosylation impacts antigen recognition and immunogenicity*. Immunology. **166**(3):380-407. doi: 10.1111/imm.13481. Epub 2022 May 4. PMID: 35416297.

[4] Fabian Higel, Andreas Seidl, Fritz Sörgel, Wolfgang Friess (2016). *N-glycosylation heterogeneity and the influence on structure, function and pharmacokinetics of monoclonal antibodies and Fc fusion proteins*. European Journal of Pharmaceutics and Biopharmaceutics, **100**:94-100, ISSN 0939-6411, https://doi.org/10.1016/j.ejpb.2016.01.005.

[5] Edwards E, Livanos M, Krueger A, Dell A, Haslam SM, Mark Smales C, Bracewell DG (2022). *Strategies to control therapeutic antibody glycosylation during bioprocessing: Synthesis and separation*. Biotechnol Bioeng. **119**(6):1343-1358. doi: 10.1002/bit.28066. Epub 2022 Feb 28. PMID: 35182428; PMCID: PMC9310845.

[6] Biskup K, Braicu EI, Sehouli J, Fotopoulou C, Tauber R, Berger M, Blanchard V (2013). *Serum glycome profiling: a biomarker for diagnosis of ovarian cancer*. J Proteome Res. **12**(9):4056-63. doi: 10.1021/pr400405x. Epub 2013 Jul 29. PMID: 23889230.

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