Absolute Quantitation of N-linked Glycans attached to Biotherapeutics with Isotopically Labeled Internal Standards

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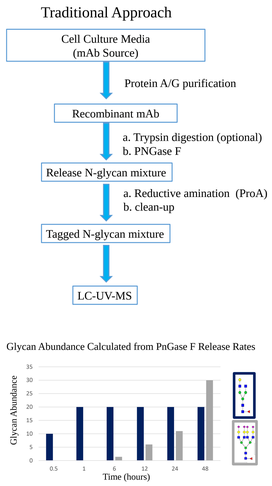
Overview

Develop isotopically labeled glycoproteins to be used as internal standards for the relative and absolute quantitation of glycoprotein glycans, particularly those attached to human IgGs.

Introduction

The glycosylation on therapeutic IgGs is known to have profound effects on various therapeutic properties, such as efficacy, serum half-life, anti-inflammatory attributes, etc.

The current methodology for monitoring glycosylation consists of a complex process involving the purification of the IgG, release/derivatization of the glycans followed by their analysis. The numerous steps introduce a variety of potential sources for experimental error. For instance, it is known that large highly sialylated glycans are more difficult to release than smaller neutral glycans causing the glycan profile to change based on the extent of the de-glycosylation procedure.[1]



In the analysis of an Erythropoietin (EPO) - IgG fusion protein, the rate of PnGase F release of the tetra-antennary fully sialylated glycans from EPO was found to be almost 400x slower than release of the bi-antennary non-sialyated IgG glycans. Kinetic analysis revealed that the half-life for the EPO glycans was 70 hours vs 11 min for the IgG glycans. Because of the difference in glycan release rates, the profile of the released glycans was found to change with increasing PNGase F digestion time.

The differences in glycan release rates infer that an isotopically labeled glycoprotein would improve the accuracy and precision of quantitative glycan analysis, and led GlycoScientific to develop a monoclonal IgG_1 antibody with a variety of isotopically labeled residues, including: $UMAb_1$-P ^{13}C and ^{15}N labeling of Lys and Arg residues, $UMAb_1$-G ^{15}N labeling of the glycans, and $UMAb_1$-GP with labeling of both the amino acids and glycans. And demonstrate the ability of these species as internal standard in the analysis of glycans attached to a biotherapeutic IgGs (adalimumab).

Experimental

Columns of HALO Penta-HILIC were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 2.7 µm diameter, shell thicknesses of 0.5 µm, and pore sizes of 90 Å. Mobile phase modifiers were obtained from Sigma/Millipore (Formic acid, Ammonium Formate). Acetonitrile was MS grade from JT Baker. Monoclonal antibody, Adalimumab, was produced in CHO cells, and provided by GlycoScientific (Athens, GA) as Protein A purified mAb, at 1 mg/mL. Trypsin digests (Promega) of the mAb were conducted in TrisHCl, pH 7.6, for 8 hours at 37°C, at an enzyme to protein ratio of 1:40.

Capillary column separations used the Dionex RSLC 3000 with a trap column, connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface operated at 3.8 kV potential. MS spectra were recorded in the Orbitrap, using 30,000 resolution scans, with CID MS/MS obtained using a Top 6 data dependent regime. MS data were analyzed in Xcalibur Quant Browser (v2.7), or transferred to Chromeleon v. 7.2 for integration. Chromatographic peak widths are reported as half height (PW1/2). Analyses of tryptic fragments employed a 0.5 mm ID x 150mm Penta-HILIC capillary column, operated at 15.0 µL/min, and 60°C. A trap column of the same material, of 2.6 µL size (0.5 mm x 12.5 mm) was obtained from Optimize, Inc. Analytical gradient conditions were from 70%B for 4min, then 70-62%B in 64 min.

Additional HILIC-SRM experiments were performed using a Halo-penta-HILIC column at 0.6mL/min and a 67-57% ACN in 0.1% formic acid and water gradient for 30 min and an ABSCIEX Q-Trap 4000 utilizing multiple reaction monitoring. The relative ratios of the glycans are obtained by SRM detection of the analyte glycoforms, and the isotopically labeled glycoforms were used as an internal standard for relative and absolute quantitation.

Use of an IgG with Isotopically Labeled Glycans as an Internal Standard

The use of internal standards is the accepted strategy to facilitate quantitation via MS. The closer the chemical properties of the internal standard to its analyte, the better it compensates for the various sources of error, and thus the most desirable internal standard is typically an isotopically labeled version of the analyte itself. Consequently, the optimal internal standard for glycoproteins is a labeled glycoprotein, which led GlycoScientific to develop an monoclonal antibody with: ^{13}C labeling of Lys and Arg residues and/or ^{15}N labeled glycans (*i*HuMAb). This enables the addition of the internal standard directly into the sample prior to processing and thus over comes systematic errors associated with parallel sample handling. As demonstrated here, *i*HuMAb can be used to provide both the relative and absolute quantitation of the Fc glycoforms.

Acknowledgement

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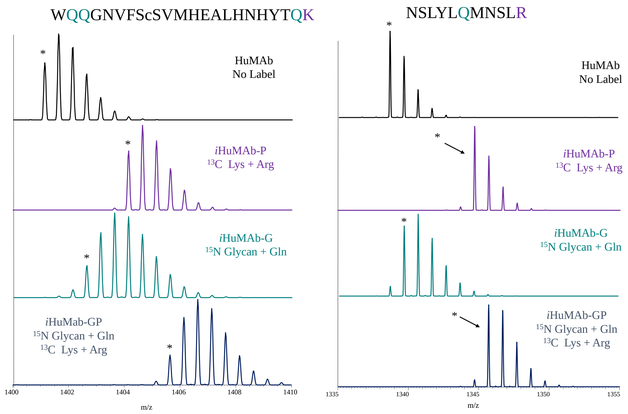
Isotopic Labeling of *i*HuMAb Peptides

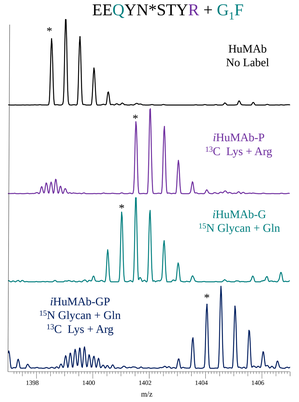
HILIC-MS experiments were performed to evaluate the level of isotopic incorporation into the various *i*HuMAb products. As can be seen from the mass spectra below of representative peptides with either a Lys or an Arg residue, incorporation of the isotopically labeled amino acids is very high (>99.5%). These spectra also demonstrate that ^{15}N intended for the glycan leaks into the peptide. The sites of ^{15}N incorporation into the peptide were found to be the amides on Gln and Asn, with the labeling efficiencies of ~ 75% and ~20%, respectively. The monoisotopic peak is denoted with a *.



Isotopic Labeling of *i*HuMAb Glycopeptides

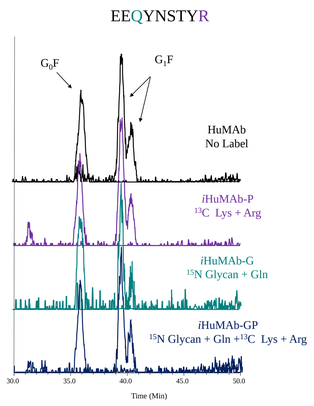
Mass spectra of the Fc glycopeptide possessing the G_1F glycan, right, demonstrate a high level of ^{15}N incorporation, estimated to be (>95%) into the N-acetyl groups of the glycan. The monoisotopic peak is denoted with a *.

The high level of isotopic incorporation make these ideal internal standards for quantitative analysis of either/both the peptide and glycan aspects of human IgGs.



Glycoform Profiling with HILIC-MS

HILIC has a long history of resolving glycan isomers.[2] We have recently demonstrated that HILIC can resolve isomeric glycopeptide glycoforms, thus necessitiating the need to release the glycans from the peptide backbone.[3] In the case of glycans attached to the FC region of human IgGs, HILIC is capable of resolving the G_1F glycopeptides, as shown to the right. This level of chromatographic separation would suggest that profiling of the IgG Fc glycans could be performed by analyzing tryptic digest of the IgG. This streamlined procedure would reduce time, cost, and minimize the number of sample handling procedures leading to improved reproducibility while decreasing experimental artifacts.



References

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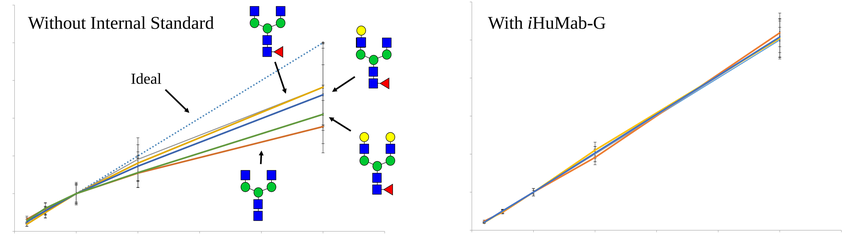
2. A. Alpert, *et al.*, *Journal of Chromatography A* **1994** 676: 191-202.
3. Y. Huang, Y. Nie, B. Boyes, R. Orlando, *J Biomol Tech.* **2016** Sep; 27(3): 98–104.

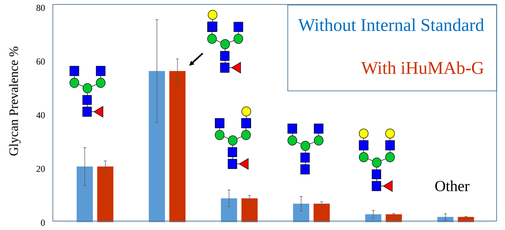
Improved Accuracy with *i*HuMAb

A serial dilution of the Adalimumab into culture media was performed to evaluate the accuracy of the approach. The result demonstrate a negative deviation from ideality when the concentration of *rh*-IgG increases, which can potentially be explained by incomplete PNGase F release as the glycans with the largest deviation from ideality have the slowest release rates.[1] The similarity of *i*HuMAb-G to Adalimumab permits this internal standard to over come these sources of systematic error and significantly improves the accuracy of the assay.



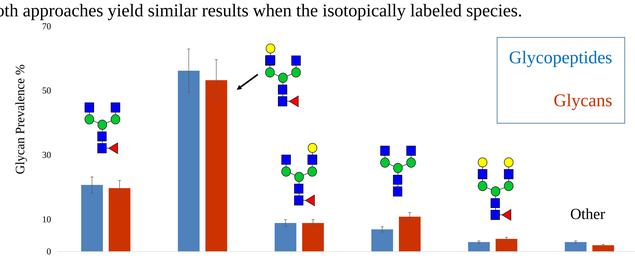
Improved Precision with *i*HuMAb

The use of *i*HuMAb-G significantly reduces the %CV compared to approaches without internal standards. This can be seen by the decreased error bars obtained when determining the glycan prevalence with the SRM glycopeptide approach. The internal standards also facilitates comparing data between different instruments and across different laboratories.



Glycopeptide vs Released Glycan

Directly analyze the glycopeptides from trypsin-digested purified protein, or even culture media would be significantly simpler than the current approach. This would obviates the need for purification and glycan release, derivatization, two of the more time-consuming steps. This approach is facilitated by the development of *i*HuMAb-P/GP and HILIC resolving glycopeptides as well as released glycans.[3] Comparison of the glycan prevalence obtained by HILIC-SRM analysis of tryptic glycopeptides and glycans released from the purified rh-IgG demonstrate that both approaches yield similar results when the isotopically labeled species.



Conclusions:

*i*HuMAb facilitates both the absolute and relative quantitation of the intact IgG and each of the glycoforms.

Use of the isotopic standard improves both the accuracy and precision because it overcomes various sources of experimental error.

*i*HuMAb can be added directly to any glycoprotein sample before any sample processing, and can be used with any glycan/glycomics work-up/analysis

The inclusion of an isotopically labeled IgG (*i*HuMAb) allow results obtained on the glycopeptides to be comparable to that obtained from released glycans.

The analysis of glyopeptides saves time and money because it is performed directly on trypsin digested cell media, without the need for IgG purification or glycan release. The reduction in steps also reduces experimental error.