Profiling the N-Glycosylation of Biotherapeutic IgGs with HILIC-MRM Analysis of Trypsin Digested Culture Media

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Overview

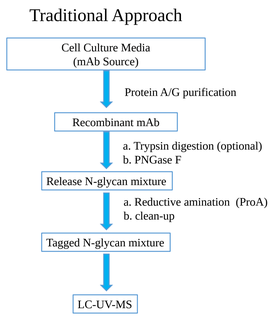
Develop a rapid procedure for the quantitation of the N-linked glycans attached to the Fc region of recombinant human IgG from direct analysis of cell culture media after trypsin digestion.

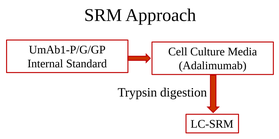
Introduction

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 know 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.

To reduce time, cost, one would like to minimize the number of sample handling procedures, which would also improve reproducibility, and decrease experimental artifacts.





We have developed a HILIC-SRM procedure that provides the N-glycosylation profile of recombinant IgGs directly from trypsin-digested culture media, and thus obviates the need for purification and glycan release/derivatization, two of the more time-consuming steps. Essentially, the sample is spiked with an internal standard consisting of an isotopically labeled IgG. The material is then reduced, alkylated, digested with trypsin, and analyzed by HILIC-SRM.

The internal standard allows for both absolute and relative quantitation across the multiple samples and reduces the experimental accuracy to <10%. To demonstrate utility our HILIC-MRM approach, we have performed a time course experiment to evaluate how glycosylation changes over the course of an expression and compared glycosylation profiles obtained from IgGs expressed under different conditions.

Experimental

A recombinant human IgG was overexpressed in CHO cells using standard expression conditions. Aliquots of cell culture media were collected, then spiked with an internal standard consisting of an IgG whose glycans are isotopically labeled. Proteins/glycoproteins in the sample are reduced with dithiothreitol, alkylated with iodoacetamide, and then digested with trypsin. The resulting peptide/glycopeptide mixture is analyzed by HILIC-MS 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, 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 a human monoclonal IgG1 antibody with ^{15}N labeled glycans (UmAb1-G), ^{13}C and ^{15}N labeled Lys and Arg residues (UmAb1-P) or both the glycans and peptide being labeled (UmAB1-GP). 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. And as demonstrated here, UmAb1 series can be used to provide both the relative and absolute quantitation of an intact IgG and the Fc glycoforms.

Acknowledgement

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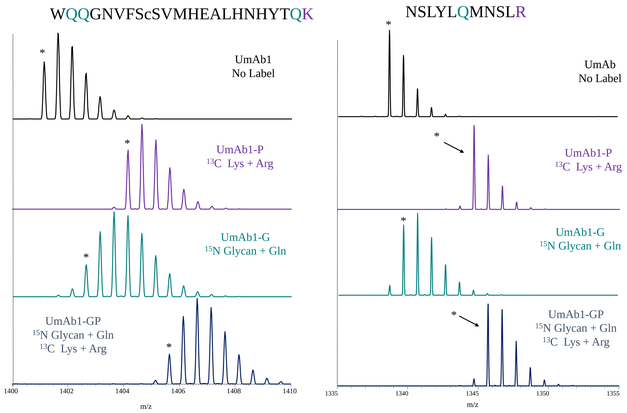
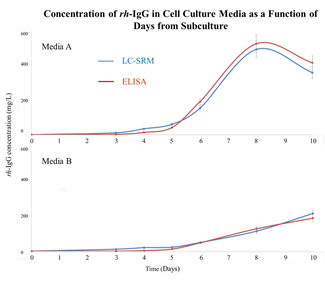
Isotopic Labeling of UmAb Peptides

HILIC-MS experiments were performed to evaluate the level of isotopic incorporation into the various UmAb1 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 *.



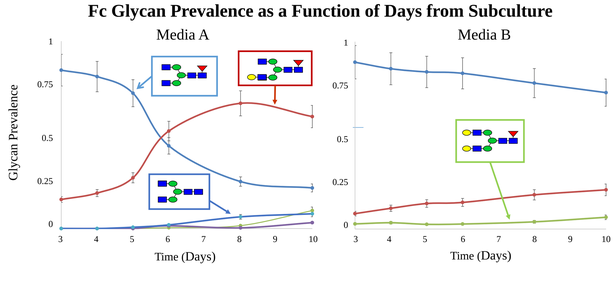
IgG Expression Levels

HILIC-SRM and ELISA were both utilized to measure the concentration of Adalimumab in the culture broth using two different culture media. These studies show that medium A provides a higher concentration of Adalimumab and that this is reached by Day 8. However, the cells have a fairly short stationary phase and have entered the decline phase by Day 10. Alternatively, Medium B shows an extended lag phase, which leads to a significantly lower concentration of the desired product. This study also demonstrates the utility of the isotopically labeled standard for the accurate determination of the absolute quantity of intact antibody, and each of the glycoforms.



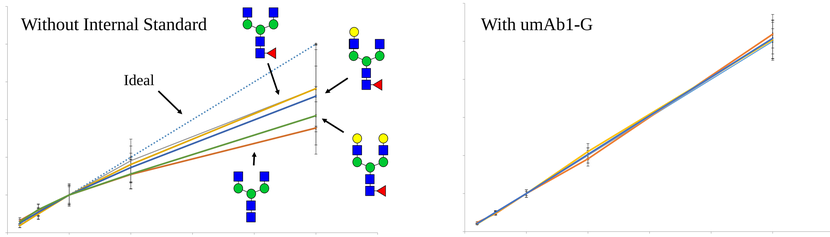
Changes in Glycan Profile

HILIC-SRM was used to quantitate each N-linked glycan attached to the Fc region as the cell culture was expanded. This study revealed that the glycan profiles changed based on how many days after sub-culturing the media is sampled. At early times, the A_2G_0F is dominant and accounts for over 85% of the total Fc glycans. As the culture is allowed to expand, the concentration of the two A_2G_1F isomers increases, however the two isomers do not increase at the same rate. The A_2G_1F isomer with the Galactose on the 3 branch appears earlier, while the isomer with the Galactose on the 6 branch lags behind. A possible explanation for this behavior is that there are two β-Galactosyltransferases responsible for adding these Galactose moieties and that these are differentially expressed as the cell density increases. At longer time points, other glycans appear.



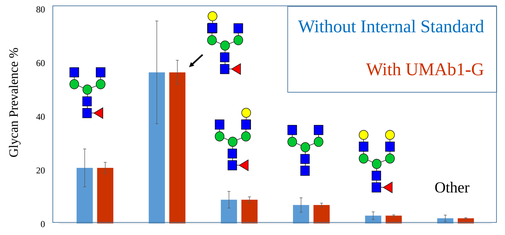
Improved Accuracy with UmAb

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 Adalimumab 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 UmAb1-G to Adalimumab permits this internal standard to over come these sources of systematic error and significantly improves the accuracy of the assay.



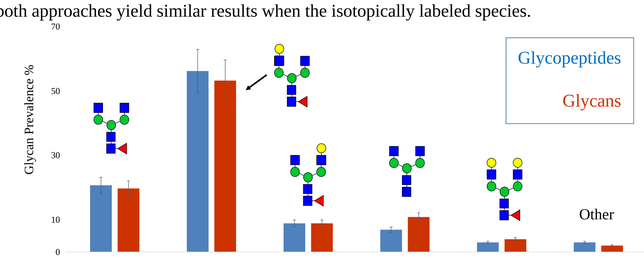
Improved Precision with UmAb1

The use of UmAb1-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 UmAb1-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 Adalimumab demonstrate that both approaches yield similar results when the isotopically labeled species.



Conclusions:

A general LC-SRM approach has been created to determine the level of 36 different glycans attached to the Fc region of an IgG. These glycans represent every glycan previously found on a human IgGs.

The analysis 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.

The inclusion of an isotopically labeled IgG (UmAb1-P/G/PG) allow results obtained on the glycopeptides to be comparable to that obtained from released glycans.

UmAb1-P/G/PG 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.

UmAb1-P/G/PG can be added directly to any glycoprotein sample before any sample processing.

UmAb1-P/G/PG can be used with any glycan/glycomics work-up/analysis