Individuality Normalization when Labeling with Isotopic Glycan Hydrazide Tags (INLIGHT™): A Novel *N*-Linked Glycan Relative Quantification Strategy

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Bioanalytical characterization and quantification of protein post-translation modifications (PTMs) is fundamental to elucidating the functions and activities of PTMs in mediating intra- and extra-cellular interactions. More importantly, how and to what degree do post-translational modifications participate in mediating human health as well as change in response to illness or disease? In a typical discovery-based, bottom-up proteomics experiment (i.e., whole proteome is digested with a proteolytic enzyme), one can often detect the presence of PTMs, including phosphorylation, glycosylation, acetylation and ubiquitination.

Biomarker-based mass spectrometry has evolved exponentially over the past 20 years, facilitating in-depth analysis of biological samples. Moreover, comparative genomics, transcriptomics, proteomics, glycomics and metabolomics have increased awareness of the integrated complexity found in all biological systems. However, to enable the detection of PTMs at a depth sufficient to understand the underlying biology and function or dysfunction of a specific protein or in a proteome-wide measurement (global proteomics), a specific PTM of interest must be approached in a targeted fashion. The challenge of distinguishing healthy and diseased populations is embraced through collaborative efforts to quantify biological change at the molecular level.

N-Linked glycosylation is known to be involved in the regulatory affairs of a proteome, weakening the ability to distinguish abnormal and normal, in terms of protein structure and function. *N*-Linked glycosylation is a very common PTM, and it has been estimated that 60-90% of all mammalian proteins are glycosylated while membrane and secreted proteins are almost always glycosylated.[1] Glycosylation is a key PTM, mediating both intra-cellular and extra-cellular communication; principally, at the protein level: regulation of folding, intra-cellular trafficking, secretion and extra-cellular recognition (e.g., cell adhesion and immune response.[2,3] These key physiological roles of glycosylation have prompted active research in what is collectively known as the field of glycomics.[4-6]

Numerous strategies may be used to study glycosylation including top-down approaches which invoke lectin affinity enrichment of
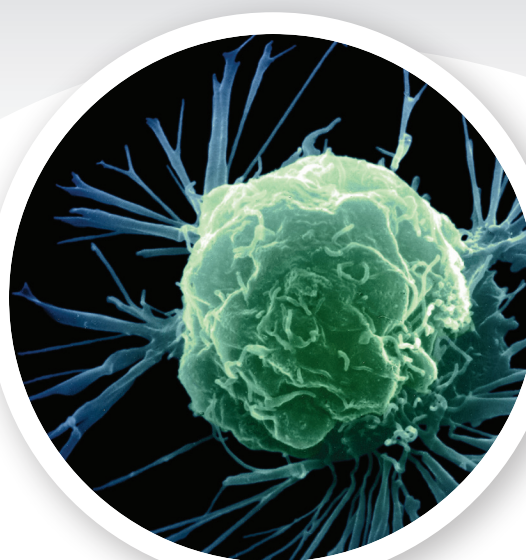
the glyco-proteome or a bottom-up approach, digesting the glycoproteome into glycopeptides that are subsequently enriched by lectin chromatography; resultant peptides are then subjected to LC/MS analysis. Alternatively whole glycans may be cleaved and isolated at the protein and/or peptide levels, thus generating a global view of the entire glycome from which the glycans are derived.[7]

There are two major types of glycans, distinguished by the amino acids they are coupled to and/or the type of covalent linkage (oxygen-linked or nitrogen-linked): *O*-linked (serine and threonine residues) and *N*-linked (asparagine residues). Both glycan types can be cleaved from their glycoconjugates chemically or enzymatically.[8] *O*-Linked glycans are commonly cleaved using chemical approaches, including hydrazinolysis or β-elimination. Intact *N*-linked glycans are commonly cleaved enzymatically using PNGase F or A.[9,10]

Typical analysis of *O*-linked or *N*-linked glycans (once they have been isolated) requires that the naturally hydrophilic and labile oligosaccharide structures be derivatized. Chemical derivatization methodologies that enable detection by mass spectrometry include permethylation, reductive amination, or hydrazone formation.[11-13] It is important to note that in instances where one can incorporate stable isotope labels via *in vivo* labeling, IDAWG is a viable approach and one that CIL also supports.[14]
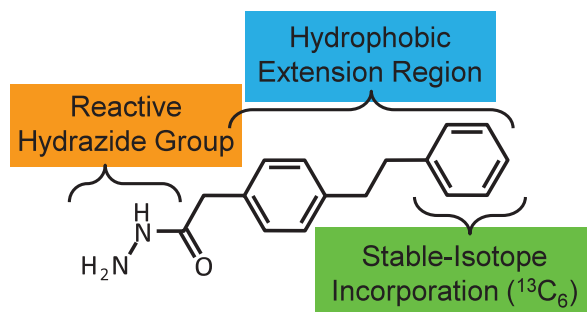
Figure 1. INLIGHT™ reagent structure; 2-(4-phenethylphenyl)acetohydrazide, available in natural or isotopically labeled forms.

The multi-functional hydrophobic, hydrazide reagents[15] included in the INLIGHT™ approach are shown in Figure 1. These tagging reagents provide three main advantages: First, the chemistry used to couple the hydrophobic tags with cleaved N-linked glycans is hydrazone formation (Figure 2), which provides efficient derivatization without requiring addition (and post-labeling removal) of salts (as required in reductive amination techniques). Second, the hydrophobic extension region dramatically improves response in electrospray ionization and allows the use of reversed-phase chromatography. Third, the ability to produce a version with a stable-isotope label ($^{13}C_6$) enables relative quantification of cleaved N-linked glycans. These were key determinants during the reagent development phase of these hydrazide tags.[15,16]

The INLIGHT™ N-glycan preparation protocol is shown in Figure 3; starting with two samples (for comparative analysis), these could be a complex biological matrix such as plasma, or biologic samples (protein therapeutic) being characterized and/or compared to a biosimilar during production and quality control. The first step is to release the N-linked glycans from the glycoproteins using the enzyme PNGase F with subsequent purification of the released glycome by solid-phase extraction (SPE). The next step is to react the N-linked glycans with the hydrazide reagent (light or heavy

form) via hydrazone formation. The two derivatized N-glycome samples are mixed 1:1 (v/v) and subjected to LC/MS analysis. Finally, data processing of extracted peak areas includes an isotopic overlap correction and normalization; ratios of each individual glycan pair reveal the differences between the two samples in a quantitative fashion.[17,18]

Results

Figure 4 illustrates how the acquired dataset is processed for the relative quantification of N-linked glycans between two samples.[17] One sample was labeled with the "light" version of the tag while the other was labeled with the "heavy" version of the tag using the workflow described in Figure 3. In this example, LC/MS analysis was performed using a reversed-phase HPLC nanoflow system coupled to a Q-Exactive Orbitrap hybrid mass spectrometer. The total ion chromatogram is shown for 25-35 minutes, demonstrating the retention and elution of a broad range of N-linked glycans. At a single retention time (see mass spectrum in the inset) one can readily resolve and observe the glycan pairs (light and heavy labeled forms of the same glycan composition). The next panel in Figure 4 shows the extracted ion chromatogram (XIC) for the corresponding glycan pair (notice that both forms co-elute with great separation efficiency). The peak area is obtained from the XIC and is corrected for isotopic overlap followed by a total glycan normalization per data acquisition (taking into account any variability in total individual glycan abundances between LC/MS datasets). Alternative data processing strategies are available, including GlycReSoft software for automated recognition of glycans detected in LC/MS datasets.[20]

Discussion

The labeling of N-linked glycans using these novel hydrazide reagents allows for efficient comparative analysis of two samples. The tags afford many benefits.

Efficient Derivatization. The coupling of the hydrazide tag with N-linked glycans cleaved from proteins via *hydrazone formation* has been optimized with a reaction efficiency of >98%.[15,16] In addition, derivatized glycome samples are analyzed *without* rigorous post-derivatization purification, minimizing sample loss and time from derivatization reaction to LC/MS analysis.
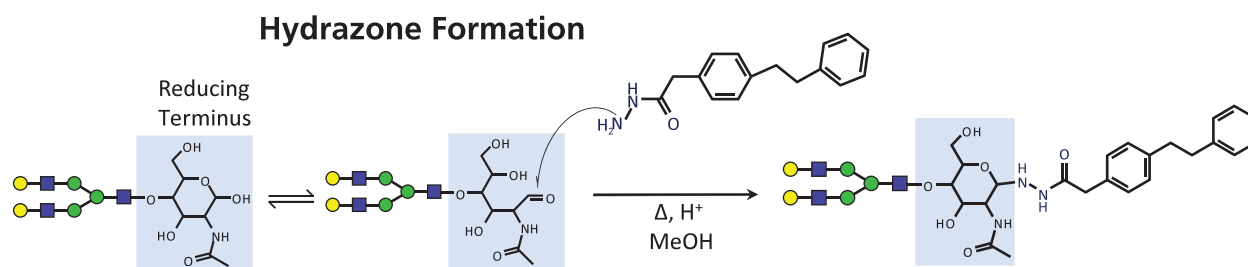

Figure 2. Proposed hydrazone formation mechanism using INLIGHT™ reagent and conditions.

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INLIGHT™ Workflow Includes:
• *N*-Linked glycan release and purification
• Incorporation of natural or stable-isotope hydrazide reagents
• Reversed phase nLC-ESI-MS data acquisition
• Generating extracted ion chromatograms (Light and Heavy)
• Peak area integration (Light and Heavy)
• Isotopic overlap correction (per *N*-glycan)
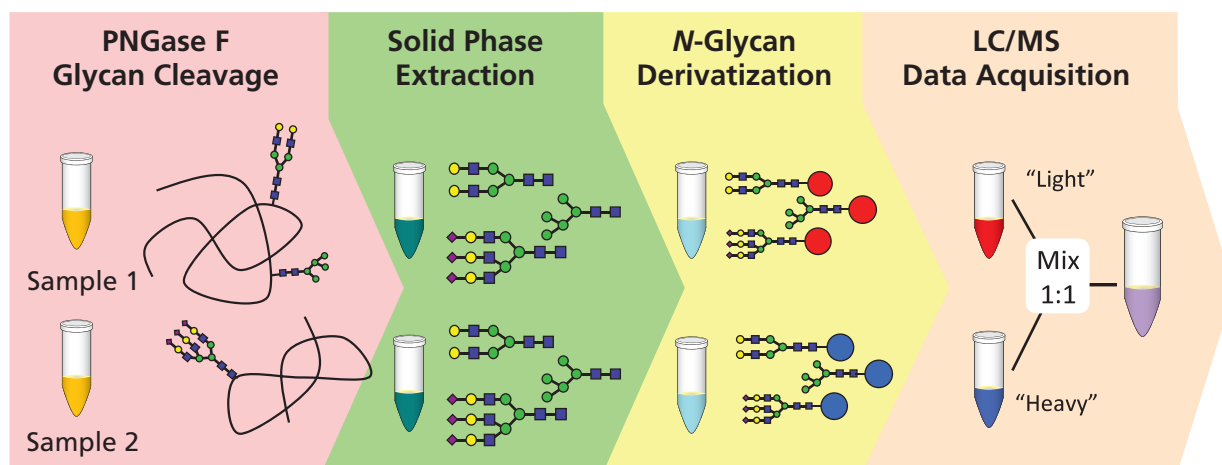• Total glycan abundance normalization (per data acquisition)
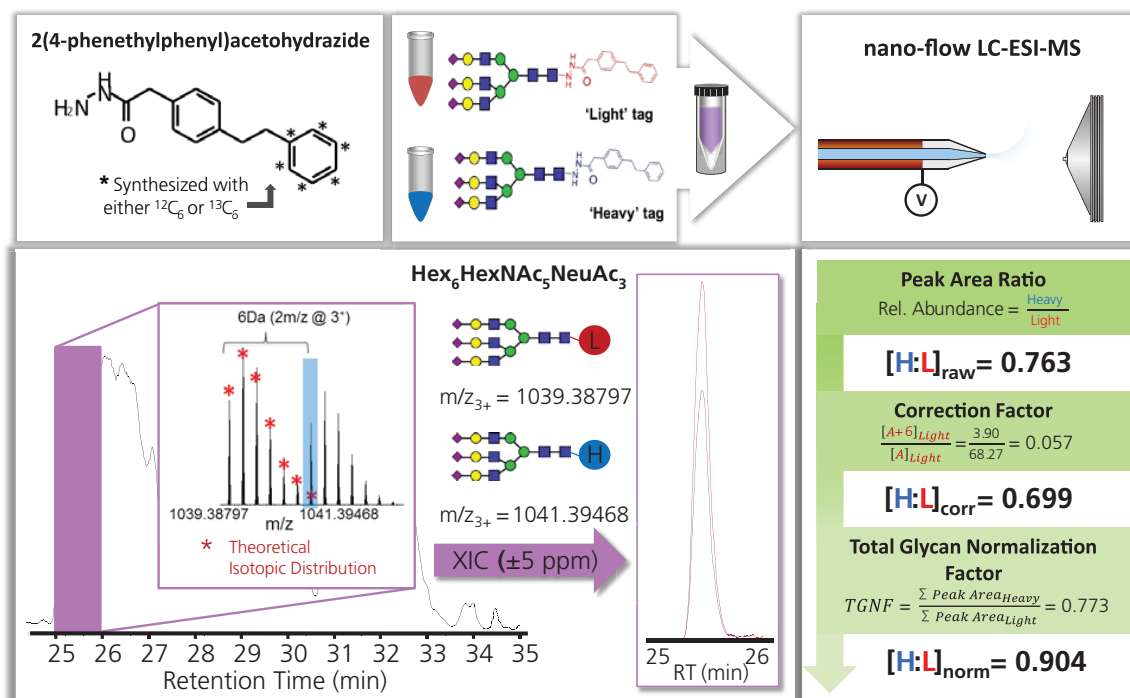

Figure 3. INLIGHT™ protocol; pre-LC/MS data acquisition.

2(4-phenethylphenyl)acetohydrazide

* Synthesized with either $^{12}C_6$ or $^{13}C_6$

nano-flow LC-ESI-MS

$Hex_6HexNAc_5NeuAc_3$

m/z_{3+} = 1039.38797

m/z_{3+} = 1041.39468

XIC (±5 ppm)

6Da (2m/z @ 3+)

1039.38797   1041.39468
m/z

* Theoretical Isotopic Distribution

25 26 27 28 29 30 31 32 33 34 35
Retention Time (min)

25   26
RT (min)

Peak Area Ratio
Rel. Abundance = $\frac{Heavy}{Light}$

[**H**:**L**]$_{raw}$ = 0.763

Correction Factor
$\frac{[A+6]_{Light}}{[A]_{Light}} = \frac{3.90}{68.27} = 0.057$

[**H**:**L**]$_{corr}$ = 0.699

Total Glycan Normalization Factor
$TGNF = \frac{\sum Peak\ Area_{Heavy}}{\sum Peak\ Area_{Light}} = 0.773$

[**H**:**L**]$_{norm}$ = 0.904

Figure 4. INLIGHT™ strategy, LC/MS data acquisition and analysis.

(continued)

Increased Hydrophobicity. As a result of increased *hydrophobicity*, an increase in glycan abundance is observed, which allows detection of lower abundant glycans. Moreover, one can now utilize reversed-phase chromatography which improves glycan separation efficiency. Note that the versatility of the method allows use of either HILIC or reversed-phase HPLC in an online LC/MS platform.[19]

Relative Quantification. The incorporation of a stable-isotope label allows for relative quantification of *N*-linked glycomes derived from two different samples. Derivatized glycan samples are processed in parallel, mixed and analyzed together eliminating instrument and ESI variability.

Collectively, the INLIGHT™ *N*-glycan tagging reagents provided in this kit (GTK-1000) are an *efficient* and *versatile* addition to the glycoscience toolbox.

Acknowledgements

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