# Increasing the hydrophobicity and electrospray response of glycans through derivatization with novel cationic hydrazides†

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**Novel tags are used to increase the hydrophobicity of glycans and impart a permanent charge yielding as great as a ~5-fold increase in electrospray response from both a standard and complex mixture.**

Chemical derivatization has long been employed in the field of mass spectrometry for various purposes. Early on in the history of chemical tagging, investigators demonstrated the use of trimethylsilyl (TMS) derivatives for improved volatility and separation of alcohols by gas chromatography.[1,2] TMS derivatization is now common practice for improved detection of analytes by GC-MS.

In the mid 1980's the development of electrospray ionization (ESI) expanded the need and purpose for chemical derivatization. With the discovery of hydrophobicity as the main driving force for electrospray response,[3] investigators have developed tags to increase the hydrophobicity of various types of biological molecules.[4–7] In addition, reports have exploited the quantitative potential of mass spectrometry using labeled and unlabeled tags for comparative peptide analysis.[8–10]

The study of the function, structure and conformation of glycans attached or released from glycoproteins, commonly referred to as glycomics, is an emerging research area with mass spectrometry becoming a powerful and prominent tool for such investigations. The analysis of mixtures is difficult as the complexity of glycans presents a significant challenge for complete characterization. Glycans are often derivatized prior to mass spectral analysis *via* permethylation[11,12] or peracetylation[12,13] to increase ionization efficiency and provide stability of labile monomers under matrix-assisted laser desorption ionization (MALDI) conditions. The main pitfall in these procedures is the significant wet chemistry steps involved which limit throughput and lead to sample loss and/or contamination. In addition, incomplete reaction efficiencies can disperse a single species into multiple $m/z$ channels, thus reducing sensitivity and complicating data interpretation.

Another potential reactive site exists on glycans that contain a reducing terminus. This site is in equilibrium between its cyclic hemi-acetal and open-ring aldehyde forms. The most common derivatization technique presently employed is reductive amination utilizing widely available primary amines. These amines react with the aldehyde functional group and form an imine intermediate. Due to the instability of the Schiff base,[14] the species is reduced *in situ* to the corresponding secondary amine. Reductive amination has been utilized to increase hydrophobicity[14,15] and incorporate a stable isotope label for relative quantification.[15] This procedure is common practice for glycan analysis by a variety of detection methods and has recently been reviewed.[16] The method suffers from low throughput due to the need to purify the glycans utilizing a solid phase extraction (SPE) step after reduction. An alternative to reductive amination for glycan derivatization is hydrazone formation between the aldehyde group and a hydrazide tag. This method offers a significant advantage due to essentially the absence of any cleanup after derivatization.[17] Thus, this chemistry is facile and carried out in both high yield and with minimal processing steps that reduce sample loss; however, this method is less common, perhaps due to the lack of many commercially available hydrazides. In this communication, we have synthesized two novel hydrazide reagents, shown in Fig. 1, both with a permanent charge and hydrophobic character. The reagents were chosen based on the need to investigate the effect of different chemical properties (straight chain *vs.* ring structure) on ESI response. These reagents provide for as great as a 12-fold increase in electrospray response of a simple standard compared to the previously reported[17] and commercially available Girard's T reagent. A correlation is provided between non-polar surface area, retention time in hydrophilic interaction liquid chromatography (HILIC), and ion abundance. Finally, ion abundances and tandem MS spectra are compared from both native and derivatized *N*-linked glycans released from plasma glycoproteins.

HILIC nanoLC mass spectrometry was performed using an Eksigent nanoLC system coupled to a Thermo Fisher hybrid LTQ-FT-ICR mass spectrometer. The column was packed in-house with TSK Gel-Amide 80 material. A vented column configuration was utilized and is described elsewhere in detail.[18] Solvents A and B were 50 mM ammonium acetate and acetonitrile, respectively. Mass spectral and HILIC nanoLC

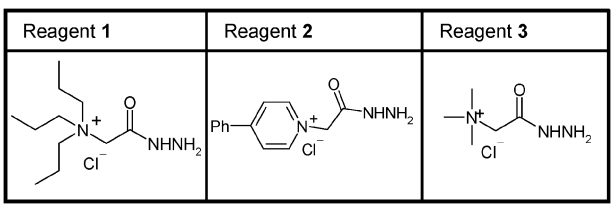


**Fig. 1** The different reagents investigated. Reagents **1** and **2** were synthesized in-house. Reagent **3** or Girard's T reagent is commercially available.

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conditions were similar to those previously reported.[19] The integrated areas under the extracted ion chromatogram (EIC) were determined by Xcalibur software version 2.0.5 and used to evaluate the electrospray response of different species.

Four 100 μL aliquots were taken from a stock standard solution of maltoheptaose (1 mg $mL^{-1}$) and pipetted into 4 Eppendorf tubes. Three aliquots were individually derivatized with the two newly synthesized reagents (**1** and **2**) and Girard's T reagent (**3**), while one was left underivatized (native species) and served as a control. Hydrazone formation was completed as previously discussed by Naven and Harvey,[17] with approximately a 20-fold excess of reagent. The samples were diluted, combined in equal volumes for an equimolar mixture and subsequently analyzed by HILIC nanoLC-MS. Calculations for determining the non-polar surface area (*i.e.*, hydrophobicity) of each tag were estimated based on bond lengths and van der Waals radii of each atom.[20]

The procedure for cleavage and purification of *N*-linked glycans from plasma was performed as previously reported.[19] For derivatization of *N*-linked glycans derived from plasma glycoproteins, a 65-fold excess of reagent to internal standard was utilized. Five μL of the glycan mixture were utilized after lyophilization and reconstitution of the solid phase extraction eluents.

Fig. 2 displays an EIC of the different maltoheptaose species from the nano HILIC-MS analysis of an equal molar mixture. In addition, the derivatives formed as a result of various tags are shown. The tripropyl derivatized species afforded approximately a 5-fold and 12-fold increase in electrospray response compared to the native species and the species derivatized with the Girard's T reagent, respectively. In addition, this species was determined both theoretically and experimentally to be the most hydrophobic, as it was calculated to have the largest non-polar surface area and was the least retained by HILIC (Table 1). Although the Girard's T reagent did impart additional non-polar surface area to the analyte, it was experimentally discovered to be the most hydrophilic (most retained) and yielded the lowest ion abundance. We hypothesize that the limited amount of hydrophobic moieties (3 methyl groups) was not sufficient to overcome the hydrophilicity imparted by the permanent charge. To further investigate this result, an equal molar direct infusion experiment was performed with all four species and it was again found the Girard's T derivative was less abundant than the native. This result, different than previously reported,[17] may be attributed to the addition of ammonium acetate to the solvent system used for both LC-MS and direct infusion experiments. The dominant ionization pathway observed was ammonium adduction. Data summarizing the non-polar surface area (*i.e.*, degree of hydrophobicity) and response ratios of the different species from both sets of experiments are summarized in Table 1. Although significant differences existed amongst the degrees of signal increase between the two sets of experiments, all species followed the same general trend (*i.e.*, the more hydrophobic the greater the signal response).

This preliminary work indicated the potential of these tags to increase glycan electrospray response; however, the ultimate goal is the application of these reagents to glycan biomarker discovery where samples are much more complex. Potential

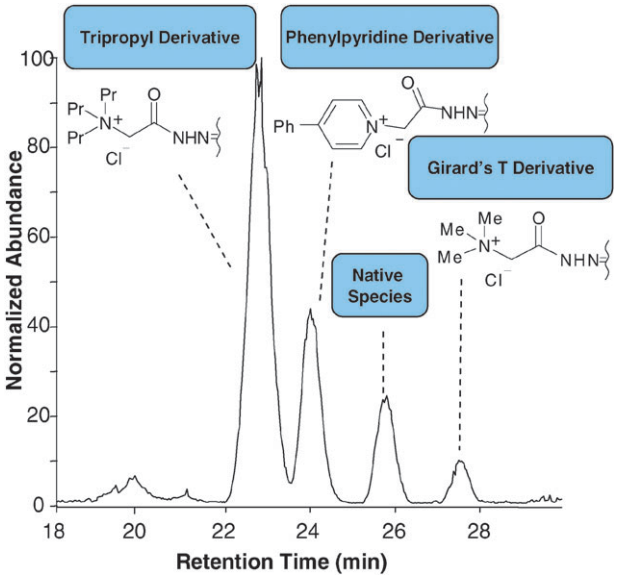


**Fig. 2** The relative responses for the analysis of an equal molar mixture of the native and various maltoheptaose derivatives.

markers for disease are most likely at relatively low abundances and any increase in electrospray response (*i.e.*, lower limit of detection) afforded by a fast and high yielding derivatization procedure would be of considerable importance. Since reagent **1** afforded the greatest ion abundance from the model experiments, we chose to investigate the utility of this reagent for derivatization of *N*-linked glycans derived from plasma glycoproteins.

Fig. 3 displays an EIC of a monosialylated glycan (native) overlaid with its tripropyl derivatized counterpart. As shown, approximately a 4.5-fold (ratio of integrated areas) increase in electrospray response was observed for the derivatized species when compared to the native. Similar to the model system, we observed a decrease in retention time which indicated an increase in hydrophobicity for the derivatized glycan.

It is possible that increasing the number of vibrational modes and/or imparting a permanent charge on the molecule, as others have previously theorized,[15] would negatively effect glycan fragmentation. Therefore, it was necessary to evaluate the effect of derivatization on glycan fragmentation *via* collision induced dissociation (CID). The inset in Fig. 3 compares the fragmentation spectra of the derivatized species to that of the native species. A high similarity exists between both fragmentation spectra. The signal-to-noise ratio of the tandem MS spectrum resulting from the derivatized species is approximately 10-fold greater compared to the native glycan. The majority of fragment peaks greater than $m/z$ 1000 contain the reducing end of this particular glycan (y-type) which explains the offset of approximately 198 Daltons, the added mass of the tag, between the derivatized and native species. Species less than $m/z$ 1000 often correspond to b-type ions or internal fragments and do not contain the reducing terminus; thus, these peaks have the same $m/z$ for both the derivatized and native species.

In this communication we have synthesized two new hydrazide reagents. Both reagents afford greater ESI response of glycans as compared to Girard's T reagent. It is clear that

 

**Table 1** Summary of maltoheptaose derivatization experiment

| Species | Tripropyl | Phpyridine | Native | Girard's T |
|---|---|---|---|---|
| Mass added/Da | 198.2 | 210.1 | — | 114.1 |
| Non-polar SA of tag/Å$^2$ | 213.3 | 131.1 | — | 99.6 |
| Retention time/min | 22.5 | 23.5 | 25.3 | 26.8 |
| LC-MS data ratio to native (integrated areas EIC) | 4.6 | 1.8 | 1.0 | 0.4 |
| LC-MS data ratio to T-Girard (integrated areas EIC) | 12.0 | 4.6 | 2.6 | 1.0 |
| Direct infusion data ratio to native (MS intensities) | 2.8 | 1.9 | 1.0 | 0.5 |
| Direct infusion data ratio to Girard's T (MS intensities) | 5.0 | 3.4 | 1.8 | 1.0 |

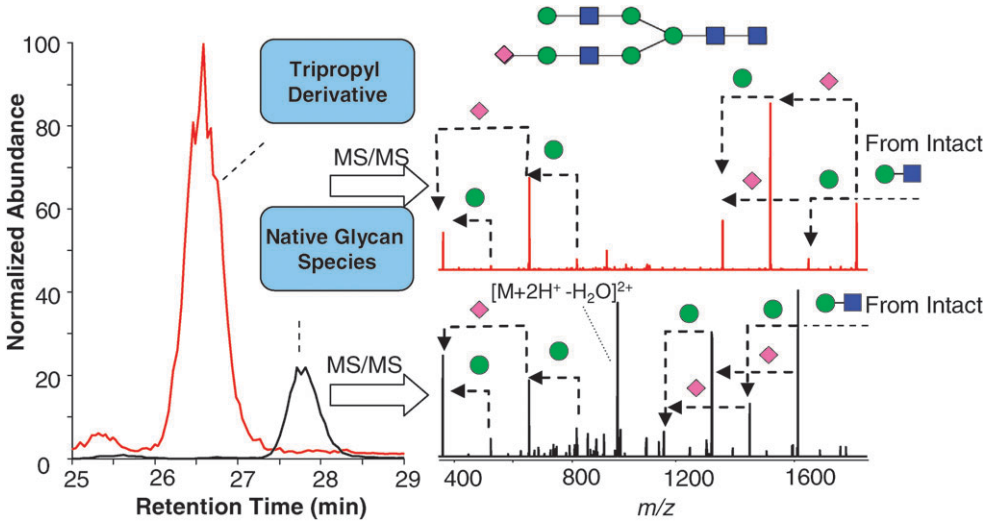


**Fig. 3** Comparison of the ESI response and fragmentation patterns of an *N*-linked glycan between native and derivatized species. The tag did not fragment or otherwise hinder tandem MS experiments. Green circle (hexose), blue square (HexNAc), purple diamond (sialic acid).

increasing the hydrophobicity of glycans significantly increases the electrospray response. Future work will explore the synthesis of a stable isotope labeled version of a hydrophobic tag which will allow for both increased sensitivity and relative quantification of *N*-linked glycans released from plasma glycoproteins.

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