Comparison of common fluorescent labels for LC-MS analysis of released N-linked glycans

John Yan, Andres Guerrero, Steven Mast, Emily Dale, Ted Haxo, Aled Jones

SUMMARY

- Analysis of N-glycans released from biotherapeutics frequently relies on the addition of a label/fluorophore.
- Using N-glycans released from MabThera and Enbrel, we compare the mass spectrometry (MS) and fluorescence response of N-linked glycans labeled with different fluorophores InstantPC, procainamide, InstantAB, 2-AB and 2-AA.
- Of the labels tested, InstantPC resulted in the brightest fluorescence and MS signal followed by procainamide, while 2-AB and 2-AA resulted in the lowest signal.
- The hydrophilic interaction liquid chromatography (HILIC) retention times and elution order of N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA are compared.
- The comparison between MS and fluorescence response shows good agreement for InstantPC labeled glycans from MabThera.
- The favorable properties of InstantPC for MS analysis allow for the detection of low abundance glycans.
- An additional benefit of InstantPC is the instant nature of the glycosylamine-reactive dye, which offers a faster workflow and time to results compared to traditional reductive amination when using other dyes.
The glycosylation pattern of biotherapeutic proteins is a critical quality attribute that impacts immunogenicity, pharmacokinetics and pharmacodynamics (1). Carbohydrates and glycans do not contain a chromophore/fluorophore suitable for online detection with standard liquid chromatography techniques (UV/fluorescence) and their MS (mass spectrometry) response is generally poor due to their low ionization efficiencies. Consequently, glycans are commonly derivatized prior to their analysis in order to increase their detectability. Traditional and commonly-used fluorescent dyes 2-AB, 2-AA (2) and procainamide (3) involve reductive amination via Schiff base formation. This approach often requires multiple steps including drying released glycans prior to labeling and a heated labeling reaction, which can take hours if not days to complete (4). The ProZyme InstantDyes like InstantPC (5) and InstantAB (6) react rapidly with glycosylamines released after PNGaseF digestion, resulting in much faster labeling and time to results (Figure 1).

To compare the fluorescence and MS response of these dyes, N-glycans from monoclonal IgG MabThera (rituximab) and Fc-fusion protein Enbrel (etanercept) were released using the GlykoPrep Rapid N-glycan Preparation system and labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA.

**Figure 1**: Comparison of Instant dye labeling and traditional reductive amination. InstantDyes react with glycosylamines released by PNGase F, while reductive amination proceeds the free reducing end hydroxyl (-OH).
Table 1: Dye Structures with fluorescence excitation (Ex) and emission (Em) wavelengths

<table>
<thead>
<tr>
<th>Labeling Fluorophore</th>
<th>λ, Ex/ Em (nm)</th>
<th>Dry Down Step</th>
<th>Labeling Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>InstantPC (5)</td>
<td>285/345</td>
<td>No</td>
<td>1 minute</td>
</tr>
<tr>
<td>procainamide (3)</td>
<td>308/359</td>
<td>Yes</td>
<td>&gt;1 hr</td>
</tr>
<tr>
<td>InstantAB (6)</td>
<td>278/344</td>
<td>No</td>
<td>1 minute</td>
</tr>
<tr>
<td>2-AB (2)</td>
<td>360/428</td>
<td>Yes</td>
<td>&gt;1 hr</td>
</tr>
<tr>
<td>2-AA (2)</td>
<td>352/435</td>
<td>Yes</td>
<td>&gt;1 hr</td>
</tr>
</tbody>
</table>

Table 1: Dye Structures with fluorescence excitation (Ex) and emission (Em) wavelengths

**RESULTS**

**UHPLC Fluorescence Performance**

- HILIC-UHPLC analysis of labeled N-glycans results in separation of the major glycans for both MabThera (Figure 2A) and Enbrel (Figure 2B).
- The fluorescence intensities of equivalent quantities of glycans loaded on column varies by the dye used for labeling (Figure 2A inset).
- Using the same UHPLC method, 2-AB-labeled glycans elute first followed by 2-AA, InstantAB, InstantPC and procainamide (Figure 2).
- Resolution differences are observed with the different dye labeled glycans. For example, only InstantPC- and procainamide-labeled samples result in the separation of both regions where Man5/Gl[6]/Gl[3] and A1F[6]/A1F[3] elute. Procainamide has the drawback of not resolving G2F/GIFS as observed with Enbrel N-glycans (Figure 2B) in addition to the observation of minor artifact peaks resulting from the developmental procainamide protocol (3).
Figure 2: A. UHPLC fluorescence profile of MabThera N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. Equal amounts of glycoprotein were prepared with GlykoPrep and labeled with each respective individual dye.
B. UHPLC fluorescence profile of Enbrel N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. Equal amounts of glycoprotein were prepared with GlykoPrep and labeled with each respective individual dye.
- The strong fluorescence signal of InstantPC compared to traditional labeling dyes 2-AB and 2-AA allows for the detection of low abundance N-glycans as observed when comparing MabThera N-glycans labeled with different dyes (Figure 3).
- The fluorescence peak area of InstantPC-labeled G0F is nearly two times the next most intense signal for procainamide (Figure 4), which requires reductive amination and a much longer sample preparation time.

**MS Performance**

- Figure 5 shows the base peak chromatogram (BPC) obtained with MabThera N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. InstantPC and procainamide-labeled N-glycans allow for the detection and resolution of low abundance glycans A1F[6] and A1F[3], while InstantAB, 2-AB and 2-AA are not baseline resolved, and do not resolve A1F[3]/[6].
- Detection of A1F [6]/[3] by MS was only observed with InstantPC and procainamide (Figure 6A extracted ion chromatogram, 6B mass spectrum for A1F [M+2H]+).

**Figure 3:** Zoomed in fluorescence profile of MabThera N-glycans labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. InstantPC and procainamide-labeled N-glycans allow for the detection and resolution of low abundance glycans A1F[6] and A1F[3], while InstantAB, 2-AB and 2-AA are not baseline resolved, and do not resolve A1F[3]/[6].

**Figure 4:** Comparison of fluorescence response. Glycans from equivalent amounts of glycoprotein were prepared with GlykoPrep, labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. Samples were analyzed by HILIC-UHPLC.

**Figure 5:** Comparison of base peak chromatograms (BPC). Glycans from equivalent amounts of MabThera were prepared with GlykoPrep, labeled with InstantPC, procainamide, InstantAB, 2-AB and 2-AA. Samples were analyzed by HILIC-UHPLC-MS.
Figure 6: 

A. Extracted ion chromatogram A1F [M+2H]^{2+}.

B. Mass spectrum of InstantPC and Procainamide labeled A1F glycan from MabThera.
CONCLUSIONS

1. InstantPC results in the highest MS and fluorescence signal of all dyes examined.

2. Glycan labeling dyes have differences in HILIC-UHPLC retention and selectivity, however retention order remains relative with InstantPC, procainamide, InstantAB, 2-AB and 2-AA.

3. InstantPC labeling of N-glycans allows for the detection and resolution of sialylated and low abundance glycans by fluorescence and MS.

4. Detection of low abundance glycan A1F [6][3] by MS was only observed with InstantPC and procainamide (Figure 6A extracted ion chromatogram, 6B mass spectrum for A1F [M+2H]2+).

5. InstantPC labeled N-glycans shows close agreement between MS and fluorescence response, facilitating relative quantification.

6. InstantPC utilizes instant chemistry allowing for much faster sample prep workflow compared to traditional reductive amination methods.

References


6. US Patent 8445292

Figure 7: Comparison of fluorescence vs MS peak areas for MabThera N-glycans labeled with InstantPC. MS peak areas represented are sum total of charge states [M+2H]2+, [M+NH4+H]2+, [M+Na+H]2+.