High Sensitivity Native Mass Spectrometry Characterization of Antibody Fluorescent Conjugates (AFC)

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**Introduction**

Antibodies conjugated with small molecule drugs are a new class of targeted therapeutics. Cysteine and lysine are two commonly used amino acids for attaching the drug through a linker. Once conjugated the challenge is determining the number of drugs linked to the antibody. Methods have been developed for the determination of DAR (average Drug to Antibody Ratio) using mass spectrometry. Here we present a rapid method derived from the work of Valliere-Douglass et al. [1] for the analysis of heterogeneous mixtures of a cysteine-linked antibody fluorescent conjugate under desalting native and denaturing conditions by LC MS.

**Experimental**

An Antibody Fluorescent Conjugate (AFC) was synthesized by adding a fluorescent tag, Alexa Fluor® 647, to the interchain cysteines of an antibody. The AFC was analyzed by LC-Q/TOF MS under native and denaturing conditions, adapted from Valliere-Douglass et al. publication. A 5 minute SEC method was developed analyzing 5-10 μg of AFC (glycosylated and deglycosylated) using a capillary flow gradient from 25 to 5 μL/min with a microflow nebulizer under native conditions. The mobile phase was 200 mM ammonium acetate, pH 7. An acetonitrile gradient using reverse phase for denaturing analysis was used, employing a C18 HPLC-Chip. Online deglycosylation was achieved using microfluidic chips with PNGase F immobilized in an enzyme reactor to characterize the deglycosylated mAb or AFC (mAb-ProtID Chip) or the released N-Glycans (mAb-Glyco Chip).

**Intact mAb and ADC Workflows and Solutions**

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**Native Size Exclusion Chromatography (SEC) Mass Spectrometry**

**Figure 1:** Native mass spectrometry of deglycosylated mAb and AFC on the 6530 QTOF. Overlay of *triplicate injections* of 10 µg of mAb (Left Top) and 15 µg AFC (Left Bottom) on column. (Right) Overlay of the deconvoluted spectra (in triplicate) using Maximum Entropy (Max Ent) with mirror plot (Upper Right Corner). Average DARs for different amounts on column (Triplicate) are as follows: 5 µg on column 3.03 with 2.43 %RSD, 10 µg on column 2.95 with 0.97 %RSD, 15 µg on column 3.32 with 3.45 %RSD.

**Figure 2:** Native mass spectrometry of intact glycosylated mAb and AFC on the 6530 QTOF. Overlay of *triplicate injections* of 1 µg of mAb (Left Top) and 5 µg AFC (Left Bottom) on column. (Right) Overlay of the deconvoluted spectra (in triplicate) using Maximum Entropy (Max Ent) with mirror plot (Upper Right Corner).

**Online Deglycosylation by HPLC-Chip: N-Glycans**

**Figure 3:** Triplicate analysis of the major N-glycans identified from the mAb using the mAb Glyco Chip with immobilized PNGase F in the enzyme reactor with porous graphitized carbon as the stationary phase on the analytical column for the characterization of the released N-glycans. N-glycans were identified using the mAb Glyco Chip Personal Compound Database Library (PCDL).
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**HPLC-Chip: Denaturing Mass Spectrometry with Online Deglycosylation**

**Figure 4:** Denaturing analysis using microfluidic chip workflows of the intact AFC and online deglycosylated AFC. (Mirror plot, Left) The intact AFC using the ProtID-Chip-150 (II) packed with Zorbax 300SB-C18 for both the enrichment and analytical columns mirrored plotted against the online deglycosylation of the same AFC using the mAb-ProtID-Chip with PNGaseF immobilized within the enzyme reactor for online deglycosylation and separation of the deglycosylated mAb with C18 for the analytical column. Zoomed deconvoluted spectrum of the Light Chain + Heavy Chain deglycosylated AFC (Bottom Right) and not deglycosylated intact AFC (Top Right).

**Figure 5:** (Left) Denaturing analysis using online deglycosylation for batch-to-batch AFC comparison. (Right) Mirror plot of the intact mAb using the ProtID-Chip-150 (II) against the online deglycosylated mAb using the mAb-ProtID-Chip. Differences are highlighted in red and by offsetting the two plots.

**Conclusions**

- A rapid 5 min method adapted from Valliere-Douglass *et al.* for the analysis of mAbs and ADCs using native mass spectrometry was developed for use on the Agilent TOFs or QTOFs.
- A 12 min denaturing mass spectrometry method with online deglycosylation was developed using the mAb-ProtID-Chip with C18 as the analytical column packing material. Alternatively for glycan analysis the mAb glyco chip with porous graphitized carbon as the analytical column was used.
- LOQs of 115-125 ng and LODs of 23-25 ng on column for IgG and AFC were achieved.

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