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Advances in LC/MS for the Characterization of Biotherapeutics

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1. Introduction

Biotherapeutics exemplified by monoclonal antibodies (mAbs) are large complex molecules that are recombinantly expressed by cellular fermentation. While the primary sequence of the protein remains the same. Post translational modifications such as glycosylation are dependent on the cell lines chosen, the fermentation media, conditions, and length of fermentation. Clipping, deamidation, oxidation, etc. may occur during fermentation, purification, and storage. Biotransformations may take place *in vivo* after administration of the therapeutic agent.

Traditionally these modifications are observed analytically by a bottom-up approach, whereby the protein is proteolytically digested with enzymes such as trypsin to generate short peptides that are much easier to separate chromatographically and characterize by mass spectrometry. This is approach is well established, reliable, and highly effective. However, the relationship of multiple modifications and heterogeneity are lost through this approach

Intact mass measurement allows direct analysis of proteins which can provide greater insights into multiple modifications within a single protein molecule. This aspect of heterogeneity may be lost when analyzing via a bottom-up approach.

The inherent difficulty of intact mass analysis of large therapeutic proteins is that they are harder to chromatograph under conditions that are compatible with mass spectrometry ion sources and the multiple charge states resulting from electrospray ionization increases the spectral complexity in addition to the underlying heterogeneity of the protein.

Reverse phase chromatography provides good resolution and peak shape while the denaturing conditions afford a more efficient and therefore sensitive ionization. Size exclusion chromatography (SEC) has lower resolution and is a non-focusing separation technique. However, this can be conducted under native conditions (1) that while less sensitive/efficient than denaturing conditions results in a smaller number of charge states at higher m/z, simplifying the spectra (***Figure 1***).

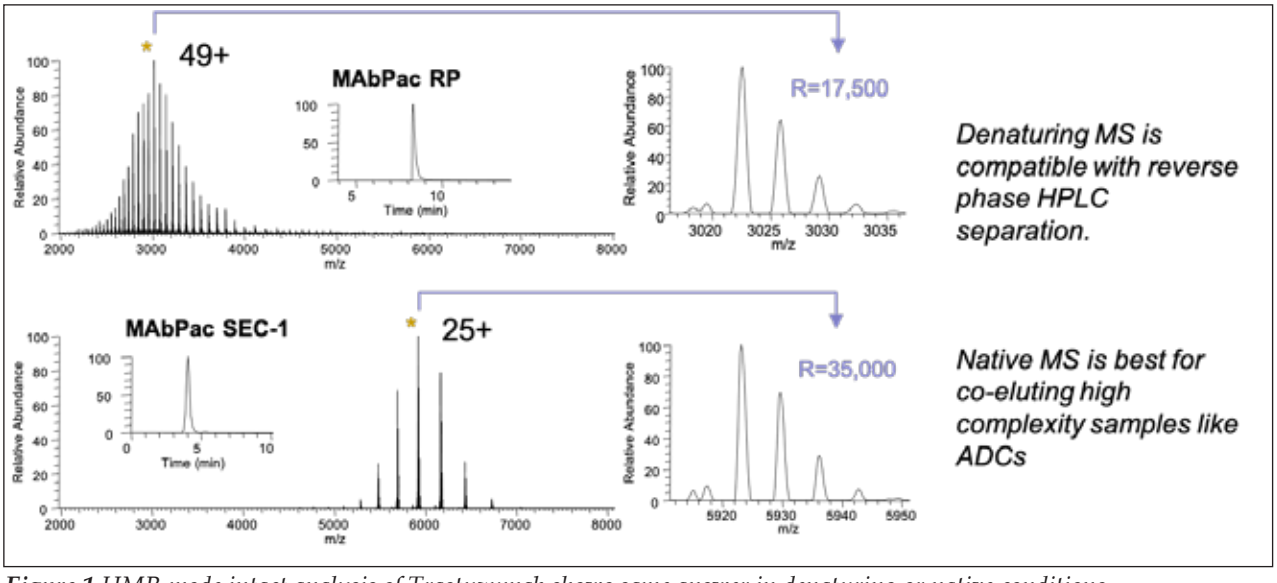

Figure 1 *HMR mode intact analysis of Trastuzumab shows same answer in denaturing or native conditions*

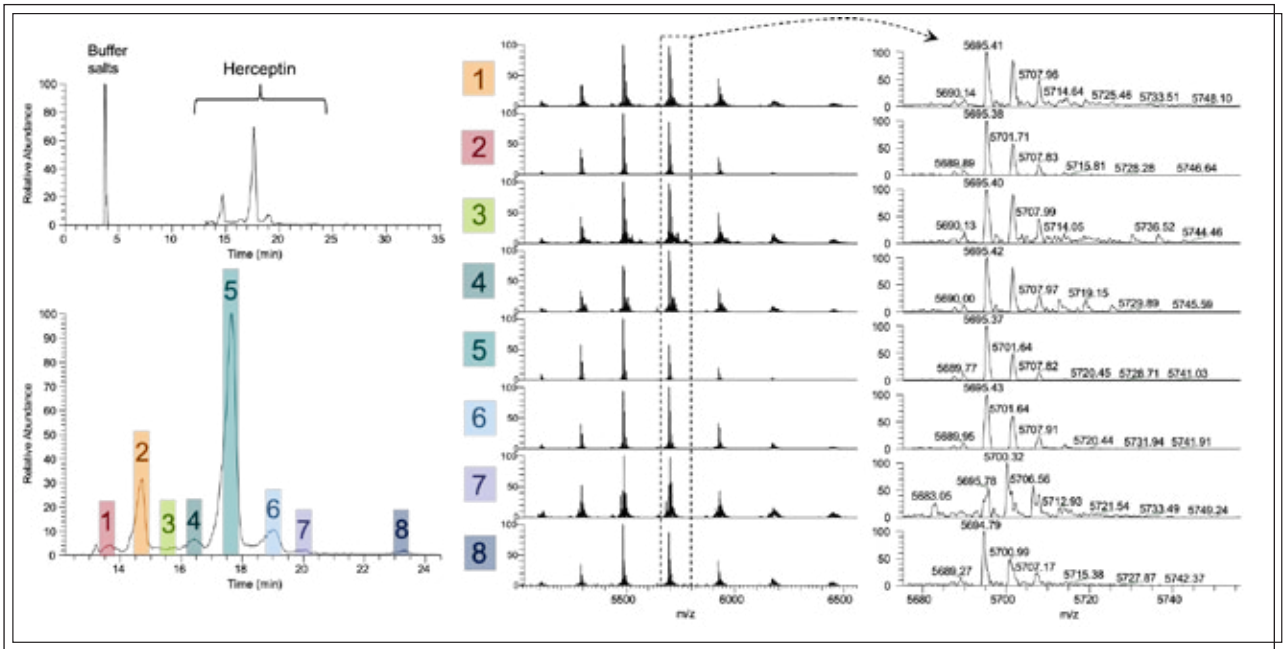

Figure 2 *Native WCX-MS: 1 uL injection of formulation Trastuzumab (21 ug/uL)*

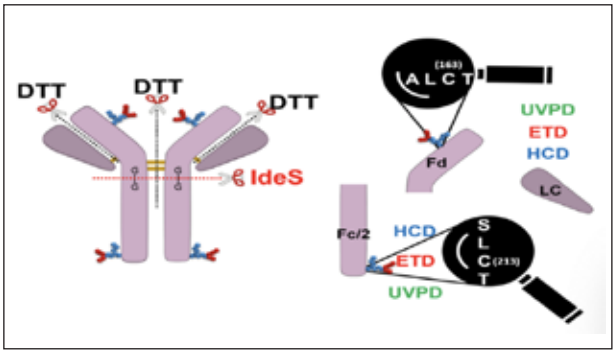

Figure 3 *Middle down analysis of a site specific ADC*

3. Results

Here, we describe charge variant native mass spectrometry (CVMS), an integrated native ion exchange mass spectrometry-based charge variant analytical approach that delivers detailed molecular information in a single, semi-automated analysis. We utilized pure volatile salt mobile phases over a pH gradient that effectively separated variants based on minimal differences in isoelectric point. Characterization of variants such as deamidation, which are traditionally unattainable by intact mass due to their minimal molecular weight differences, were measured unambiguously by mass and retention time to allow confident MS1 identification. We demonstrate that efficient chromatographic separation allows introduction of the purified forms of the charge variant isoforms into the Orbitrap mass spectrometer. Our CVMS method (2) allows confident assignment of intact monoclonal antibody isoforms of similar mass and relative abundance measurements across three orders of magnitude dynamic range (***Figure 2***).

To further characterize intact antibodies by MS/MS sequencing is still a significant challenge. On the journey to that goal, we demonstrate here the ability to determine the location of conjugation of a site-specific antibody drug candidate by middle down sequencing (3).

High sequence coverage is achieved through the use of multiple fragmentation mechanism. Namely High Eneergy Collision Induced Dissociation (HCD), Electron Capture Dissociation (ETD) and UV Photo Dissociation (UVPD) (Figure 3).

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