

# CHARACTERIZATION AND PEGYLATION SITE DETERMINATION OF A PEGYLATED BIOTHERAPEUTIC BY CID MS/MS COUPLED WITH ION-MOLECULE REACTIONS

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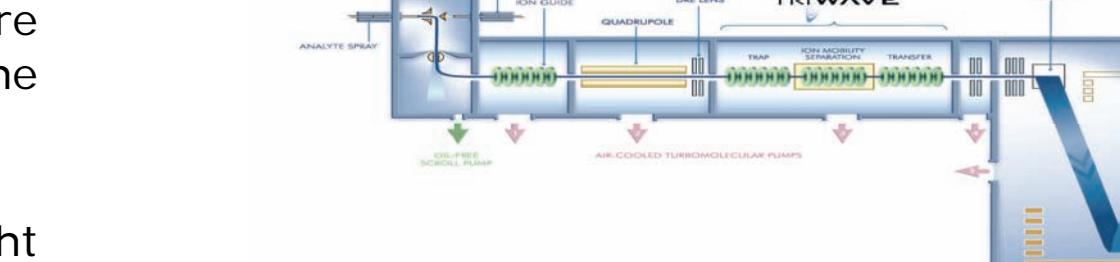
## OVERVIEW

- Polyethylene glycols (PEGs) and its derivatives are widely used in the biopharmaceutical industry for the delivery of therapeutic drugs.
- Mass spectrometric analysis of high molecular weight PEGs and PEGylated biotherapeutics is very challenging due to the great complexity and diversity of the materials.
- The use of gas phase ion-molecule reactions for the analysis of PEG, PEGylated proteins and peptides has precedent within the biopharmaceutical industry today<sup>1</sup>.
- This presentation describes: 1) An improved method to accurately measure the average molecular weight of PEGs, PEGylated peptide and protein products using ion-mobility time-of-flight mass spectrometry coupled with gas-phase ion-molecule reactions; and 2) An improved methodology to elucidate the PEGylation sites in PEGylated-biotherapeutics by sequential in-source fragmentation and CID MS/MS.
- The method is developed based on a simple and flexible modification to a Waters SYNAPT® HDMS™ System with off-the-shelf components, thus enabling a gas-phase ion-molecule reaction to be effectively coupled to the high-performance tandem mass spectrometer without sacrificing any high performance attributes of the original instrumentation<sup>2</sup>.



**MS Conditions**  
MS System: Waters SYNAPT HDMS  
Ionization Mode: ESI Positive  
Capillary Voltage: 3.0 kV  
Cone Voltage: 40 V  
Source Temp: 100 °C  
Desolvation temp: 250 °C  
IMS Cell Pressure: 0.5 mbar (N<sub>2</sub>)  
Wave Pulse Height: 5-14 V  
RF Amplitude: 320 V (Peak-to-Peak)  
IMS Cell Velocity: 240 m/s

## ADDING ION MOBILITY TO A Q-TOF MS



- The ion-mobility section is comprised by three Traveling Wave-enabled Stacked Ring Ion Guides (SRIG).
- The TRAP ion guide is used to accumulate ions and release them as packets for ion mobility separation.
- The TRANSFER ion guide conveys the mobility separated ions to the oa-TOF for mass analysis.
- Gas phase ion-molecule reactions can take place either in the TRAP cell or in the TRANSFER cell.

## SYSTEM CONFIGURATION FOR ION/MOLECULE REACTIONS

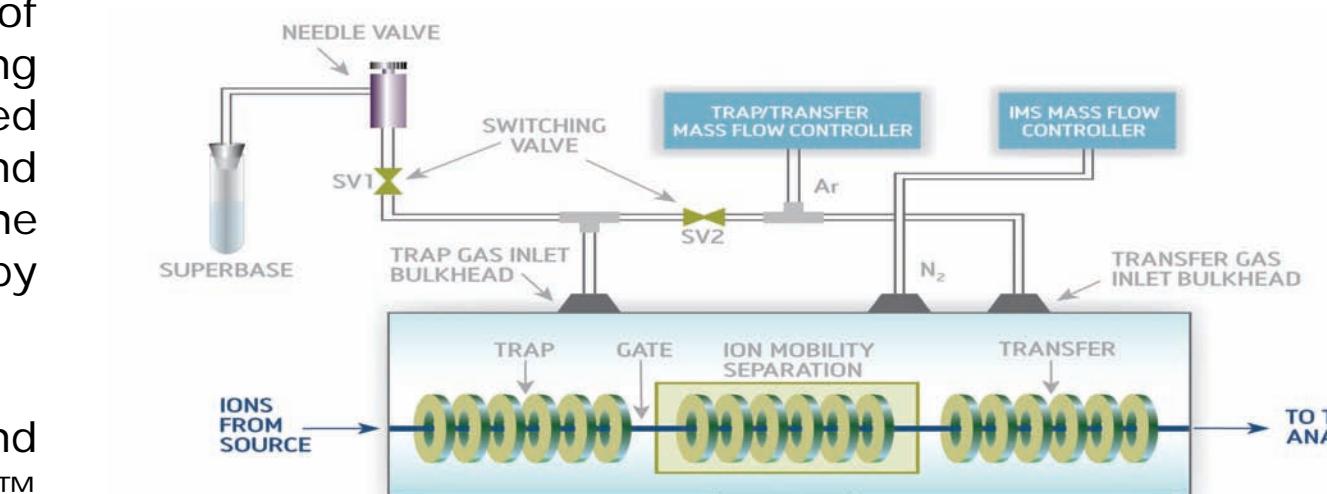


Figure 1. Instrument (SYNAPT HDMS) schematic illustrating the Triwave™ Technology embedded in a high-performance oa-TOF tandem mass spectrometer (A). Schematic diagram showing the modified gas line configuration for performing ion/molecule reaction inside the TRAP cell of SYNAPT HDMS Mass Spectrometer (B).

## ESI-TOF ANALYSIS OF PEG 4450

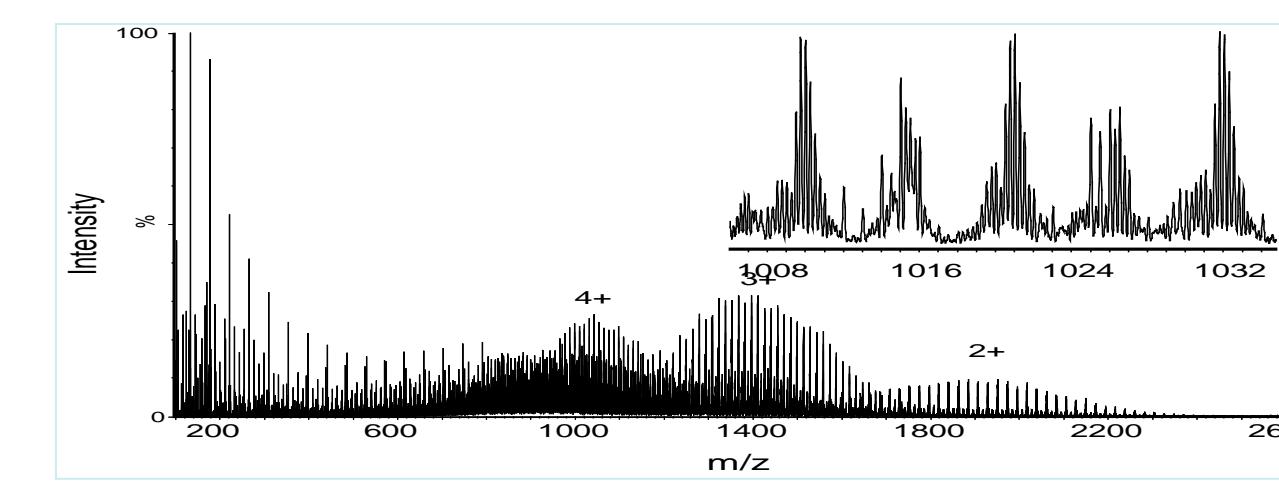


Figure 2. ESI-TOF mass spectrum of PEG 4450 without IMS separation. The isotopic resolution for peaks in the spectrum (inset) showing the sufficient resolving power of the instrument at the collected m/z window. The isotopic resolution permits the determination of the number of charges that each oligomer holds and their charge states distribution in the spectrum. Spectrum contains several charge states ranging from 2+ to 4+ that are due to the addition of multiple cations (e.g., Na<sup>+</sup>, K<sup>+</sup>, and H<sup>+</sup>) to each PEG oligomer, generating many different ion series in the spectrum. Consequently, the average molecular weight cannot be readily determined.

## ESI-IMS-TOF ANALYSIS OF PEG 4450

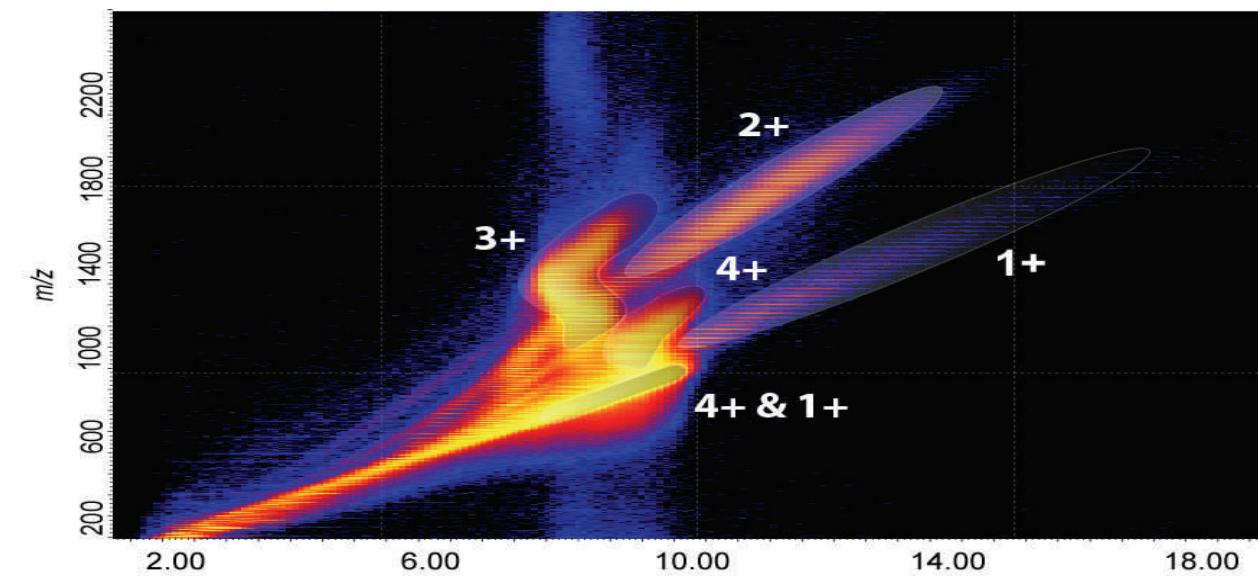


Figure 3. ESI IMS TOF analysis of PEG 4450 using SYNAPT HDMS. Driftscope™ shows the gas-phase separation power of SYNAPT in the analysis of PEG 4450. Components with different charge states (1+ to 4+) are separated via ion-mobility, thus enabling the examinations of different (minor) components in the PEG materials.

## Charge Stripping Analysis of PEG 4450

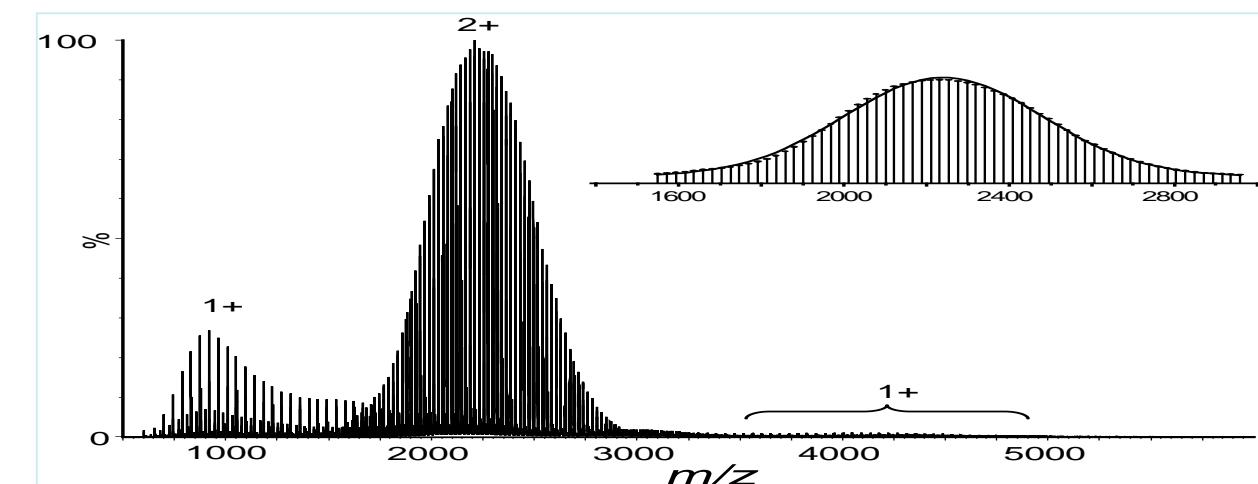


Figure 4. ESI-TOF mass spectrum of PEG 4450 after reaction of all charge states in the trap cell of the instrument with super base, 1,8-diazabicyclo[5.4.0]-undec-7-ene (DBU). The inset shows the expanded view of the best fit to a Gaussian distribution.

## Charge Stripping Analysis of PEG 20kDa

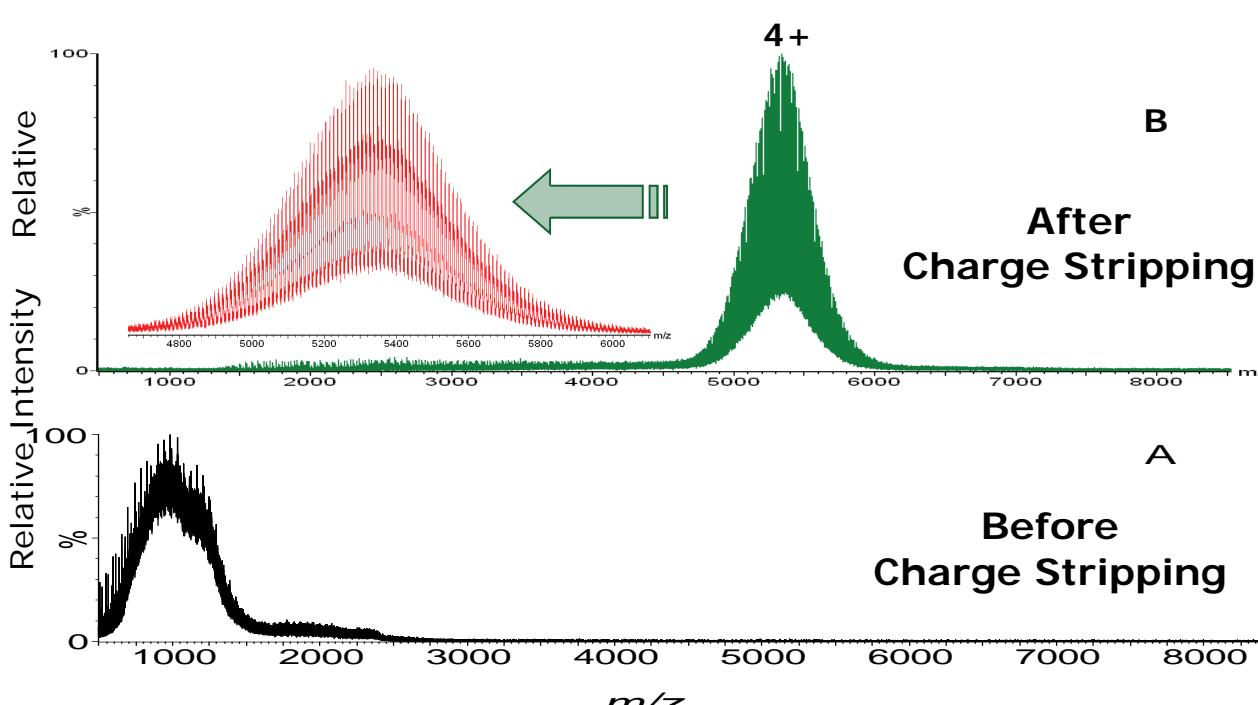


Figure 5. ESI-TOF mass spectrum of PEG-aldehyde 20 kDa before (A) and after (B) reaction with super base, DBU, in the TRAP cell of the Triwave. Reaction conditions were optimized to strip all the charge

## LC-MS PEPTIDE MAPS FROM NATIVE AND PEGYLATED GROWTH HORMONE

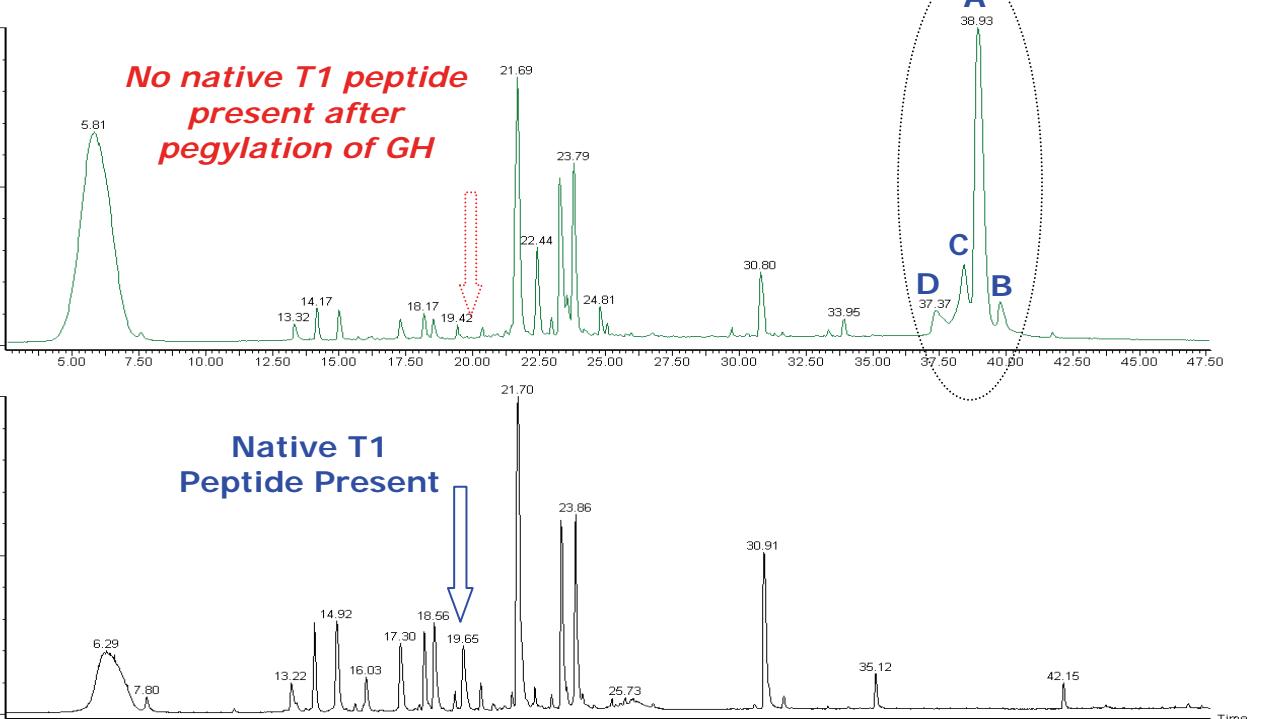


Figure 6. LC-MS peptide mapping of tryptic digest from native and PEGylated growth hormone.

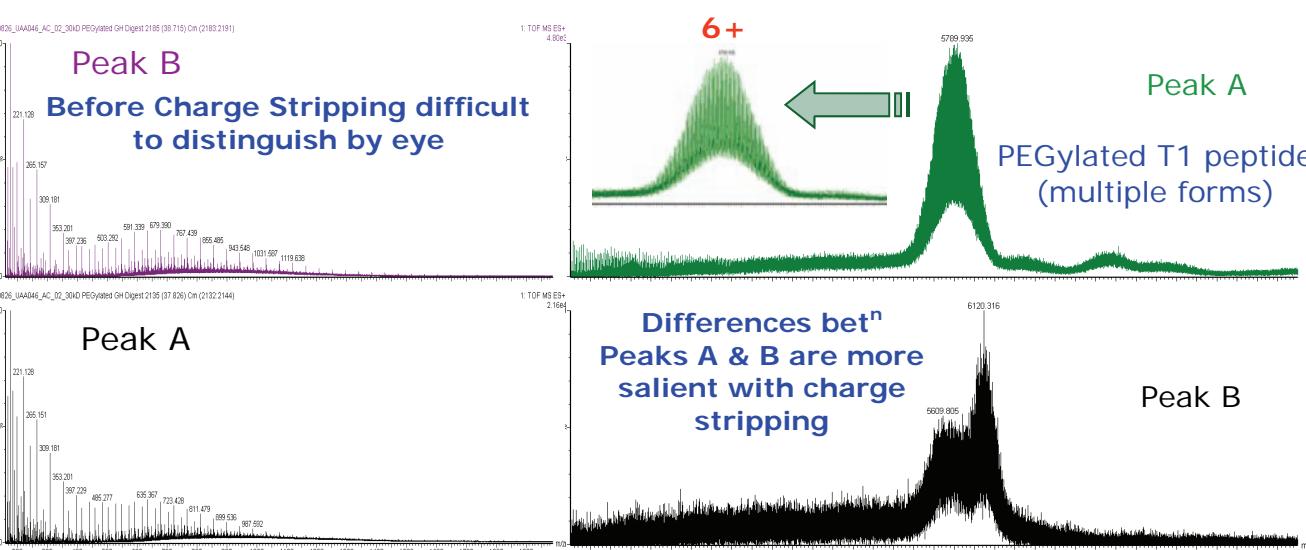


Figure 7. Mass spectra from PEGylated peptides from a regular LC-MS analysis and from a LC-MS analysis coupled with charge stripping (DBU).

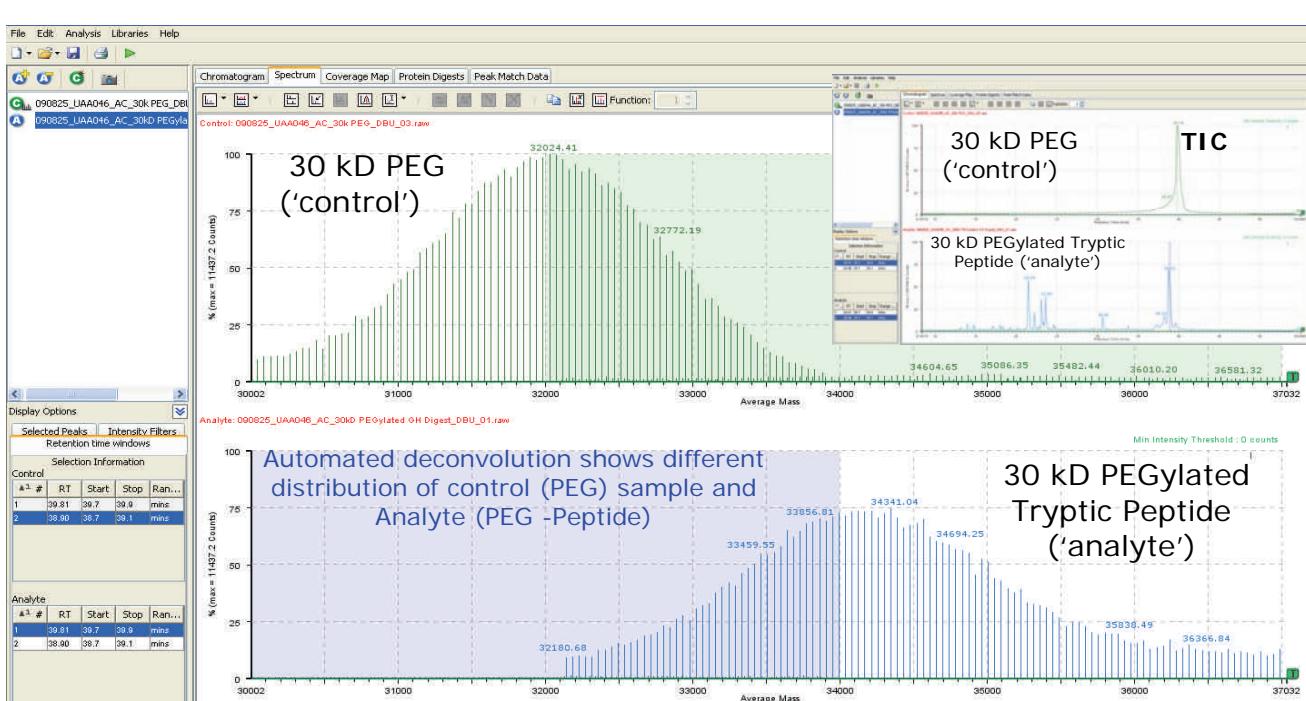


Figure 8. BiopharmaLynx™ Software view of TIC (After Charge Stripping, inset). BiopharmaLynx processed (MaxEnt 1) deconvoluted 30kD PEG and PEGylated Tryptic fragment.

## Determination of PEGylation Site by MS/MS

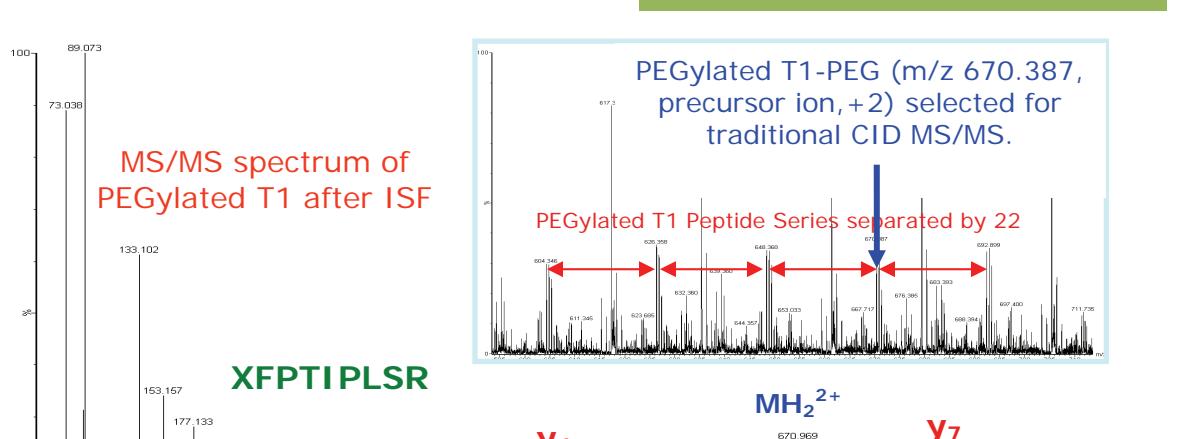
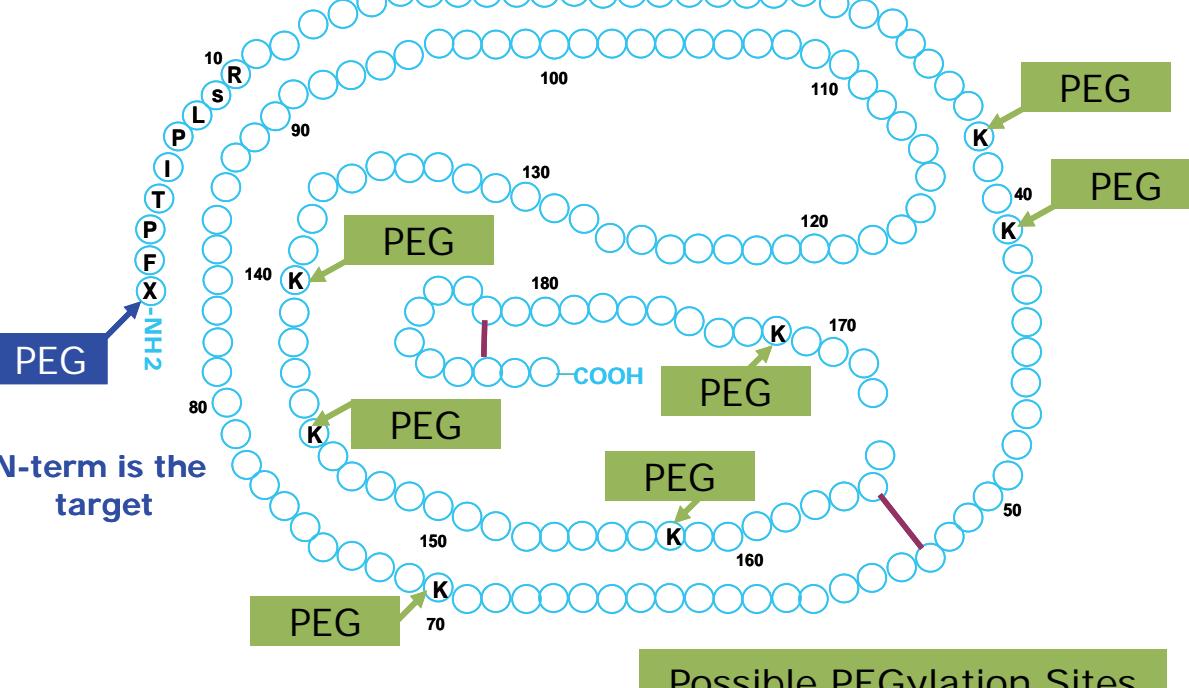


Figure 9. MS/MS spectrum of PEGylated growth hormone after in-source fragmentation (ISF) confirms PEGylation at the N-terminal. The m/z 670.387 (+2) of the precursor ion was selected for CID.

## CONCLUSIONS

- We have demonstrated a method to accurately measure the average MWs and MW distributions of large PEG using ion-molecule reaction inside a Waters SYNAPT HDMS Mass Spectrometer.
- The combination of in-source fragmentation and traditional CID MS/MS has provided a mechanism for confirming the PEGylation site of the PEGylated growth hormone.
- With many unique capabilities of the instrument, such as ion-mobility separation, the ability to perform ion-molecule reaction inside SYNAPT HDMS has truly expanded the applicability of the instrument. It is conceivable that this configuration can be readily applied to many challenging analytical tasks in pharmaceutical and biopharmaceutical industries.