Intact Mass Analysis of PEGylated Therapeutic Proteins using TripleTOF® system.

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PEGylated proteins are a rapidly growing class of biopharmaceutical products due to the enhanced pharmacokinetic drug properties and reduced immunogenicity. The poly-(ethylene glycol) (PEG) is the most commonly used polymer for covalent conjugation of proteins because it does not introduce any undesirable side effects and is approved by the US FDA. Extensive characterization of the final product (pegylated biotherapeutic) and raw material (polyethylene glycol (PEG)) is necessary for manufacturing consistency given the heterogeneous nature of commercial PEGs. However, analysis of the PEGylated biomolecules remains a formidable problem due to the molecular weight polydispersity of PEG, site of addition and number of attached PEGs. The molecular weight distribution of PEGs coupled with their inherent nature of acquiring multiple positive charges leads to mass spectral congestion creating real challenges for the mass spectrometric analysis of these molecules.

This technical note describes the LCMS method using AB SCIEX TripleTOF® System for two commonly available PEGylated biopharmaceuticals i.e Granulocyte Colony Stimulating Factor (GCSF) and Interferon (IFN) conjugated with 20KDa & 40KDa PEG respectively. The high quality MS data using TripleTOF® system yielded the accurate mass of the PEGylated proteins and also provided the additional details about molecular weight distribution and polydispersity.

Key Benefits of TripleTOF® System for Analysis of Intact PEGylated Therapeutic Proteins

1. High mass accuracy and high resolution TOF MS data provides high confidence in intact mass identification.
2. Powerful Bayesian protein reconstruction tool in Bioanalyst software, which provides accurate mass assignment even for highly heterogeneous PEGylated proteins which have a molecular weight distribution.
3. The AB SCIEX DuoSpray™ source provides highly efficient desolvation and ionization for such complex molecules.
Experimental Design:

Sample Preparation
The protein biotherapeutics standards (from Lupin limited) were desalted with 10KDa Amicon Ultra-0.5 Centrifugal Filter Units using 10% acetonitrile with 0.1% Formic acid. Depending on the salt and detergent content, the desalting was repeated 3-8 times with the above buffer.

LC Conditions
- **LC System**: Shimadzu UFLCXR Prominence 20ADXR
- **Analytical column**: Phenomenex Aeris WIDEPORE, C4, 150 x 2.1 mm, 3.6 um
- **Analytical flow**: 0.300 ul/min
- **Mobile Phase A**: Water (0.1 % formic acid)
- **Mobile Phase B**: Acetonitrile (0.1 % formic acid)
- **Mobile Phase C**: Water: ACN (50:50) with 0.2%, 0.5 & 1% Triethylamine at 0.200ul/min
- **Oven Temperature**: 60°C

Gradient Conditions:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile phase B%</th>
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<tbody>
<tr>
<td>0.00</td>
<td>5</td>
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<tr>
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<td>50</td>
</tr>
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<td>12.00</td>
<td>50</td>
</tr>
<tr>
<td>16.00</td>
<td>5</td>
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Mass Spectrometry Conditions:
- **MS System**: TripleTOF® system with DuoSpray™
- **Source**: ESI with Positive Mode
- **TOF MS range**: m/z 1500-7500 for PEG-GCSF, m/z 2000-10000 for PEG-IFN

Data Processing: Spectral deconvolution and mass reconstruction including modeling was performed using the Bayesian Protein Reconstruct tool in BioAnalyst™ V.1.5.1 Software

Intact Protein Analysis of PEGylated Therapeutic Proteins

Results and Discussion
The high quality MS data using TripleTOF® yielded the accurate mass of the Non-PEGylated as well as their PEGylated counterparts. The TOFMS data was deconvoluted using the powerful Bayesian protein reconstruction tool in BioAnalyzer software, which provides accurate mass assignment even for highly heterogeneous PEGylated proteins. The intact molecular weight of the Non-PEGylated GCSF and IFN after deconvolution is approximately 18.8 KDa and 19.3KDa respectively (Figure 2 & 3).

![Figure 2: TOFMS spectra (A) and Deconvoluted Mass Spectra (B) of GCSF](image)

![Figure 3: TOFMS spectra (A) and Deconvoluted Mass Spectra (B) of IFN](image)
The TOFMS spectra of the PEG-GCSF and PEG-IFN without TEA addition did not show any ionized molecules at the m/z ranges expected (Figure 4a & 5a). However, with the post column TEA addition, the mass spectrum showed the presence of various charge state envelopes ranging from +7 to +12 for PEG-GCSF and +7 to +10 for PEG-IFN (Figure 4(b-d) & 5(b-d)). The 20KDa PEG-GCSF ions(m/z) were mainly distributed in the 2000-7000 (m/z) range and 40KDa PEG-IFN were mainly distributed in the 5000-9000 (m/z) range in TOFMS. The post column addition of amines is known to reduce the charge complexity of the PEGylated proteins to yield a simplified and interpretable spectrum. It was also observed that the concentration of TEA affects the profile of the TOFMS spectrum. The results showed that more than 0.2% TEA is required for optimum ionization. The TOFMS spectra were deconvoluted and fitted to a Gaussian distribution using BioAnalyst™ Software. The average MW of the PEGylated GCSF and IFN were found to be in the range of 38-42 KDa and 58-63 KDa respectively with the 5-6KDa polydispersity of PEG, which is well in agreement with the theoretical mass (Figure 6 & 7).

Figure 4: TOF MS spectra of 20KDa PEG-GCSF with (a) No TEA, (b) 0.2% TEA, (c) 0.5% TEA and (d) 1% TEA.

Figure 5: TOF MS spectra of 40KDa PEG-IFN with (a) No TEA, (b) 0.2% TEA, (c) 0.5% TEA and (d) 1% TEA.

Figure 6: Deconvoluted MS spectra of PEG-GCSF at different concentration of TEA. Zoomed view of 44 Da mass difference for each ethylene glycol unit.
The expanded view of the deconvoluted spectra of the PEG-GCSF clearly showed the 44Da difference attributed to ethylene glycol units of PEG (Figure 6, 7).

**Conclusion:**

1. The TripleTOF® System provides an excellent solution for the characterization of intact PEGylated therapeutic proteins with great robustness.

2. Post column infusion of amines reduces the charge complexity and enhances the mass spectrum quality of the PEGylated proteins.

3. Molecular Mass and the mass distribution of the PEGylated protein can easily be determined from the high quality MS data using the sophisticated mass reconstruction tool in BioAnalyst™ Software.

**References:**


2. Analysis of Inact and Reduced Therapeutic Monoclonal Antibodies using the TripleTOF® 5600 System. ABSCIEX Technical Note.