Isolation and Characterization of Product-Related Variants in Biotherapeutics

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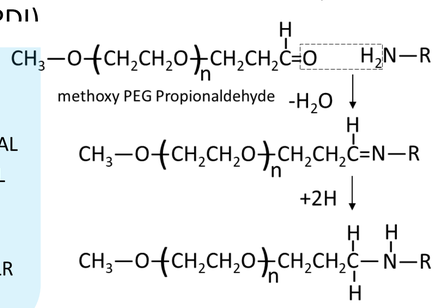
Biotherapeutics comprise a diverse class of protein-based drugs with intricate structural and functional characteristics. They exhibit inherent heterogeneity arising from various sources, including manufacturing processes, formulation conditions, and storage. Comprehensive understanding and characterization of these product-related variants are important to ensure the safety, efficacy, and quality of biotherapeutics, as well as for timely regulatory approval. Characterization typically involves isolating charge, size, and hydrophobicity variants, and identifying and quantifying their relative abundances in the drug product. This is achieved using advanced analytical tools such as semi-preparative chromatography, size- and charge-based HPLCs technologies, mass spectrometry (MS), CE-SDS, among others. Additionally, functional assessments are performed using cell-based assays and binding assays to evaluate the impact of variants on bioactivity. Our work highlights various techniques employed to elucidate the complexity of product-related variants in biotherapeutics, with a primary focus on a pegylated cytokine and monoclonal antibody.

Intact Mass Analysis of a PEGylated Cytokine

PEGylation of biotherapeutics increases their molecular weight (MW), resulting in reduced kidney clearance and protection from proteolytic degradation. PEGs are polymers formed by polymerization of ethylene glycol, consisting of ethylene oxide (C_2H_4O) repeating units. Primary structure analysis involves determining the intact molecular mass of the PEGylated protein. RPLC-MS method, with post-column addition of triethylamine, provides both the MW and distribution, known as the polydispersity index (PDI).

M[1]TPLGPASSL PQSFLLK[17]CLE
QVRK[24]IQGDGA ALQEK[35]LCATY
K[41]LCHPEELVL LGHSLGIPWA PLSSCPSQAL
QLAGCLSQLH SGLFLYQGLL QALEGISPEL
GPTLDTLQLD VADFATTIWQ
QM[122]EELGM[127]APA LQPTQGAM[138]PA
FASAFQRRAG GVLVASHLQS FLEVSYRVLR
HLAQP



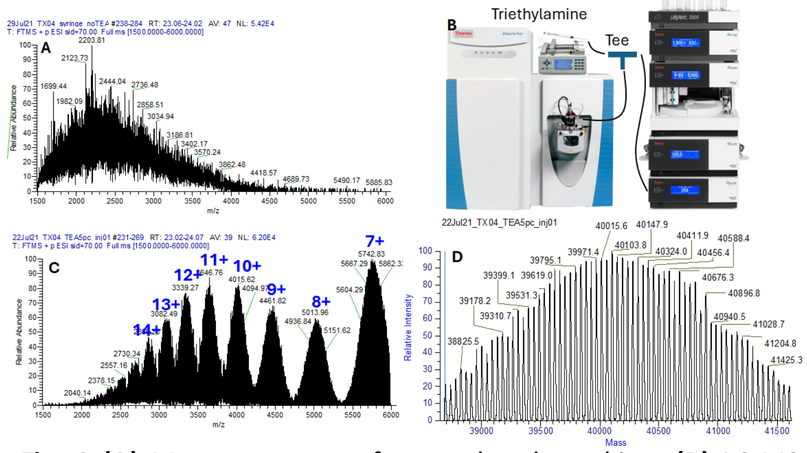


Fig. 1 (A) Mass spectrum of a pegylated cytokine, **(B)** LC-MS setup with post-column addition of triethylamine via a syringe pump, **(C)** Mass spectrum of a pegylated cytokine with 5% TEA infusion at 5 µL/min, and **(D)** Deconvoluted zero-charge spectrum obtained by combining charge state distributions shown in panel C. The average MW of the pegylated cytokine ranges from approximately 38.7 to 41.7 kDa. The mass error of the cytokine conjugated with PEG containing 483 ethylene oxide (EO) units is 0.7 Da. (Theoretical MW of PEGylated cytokine with 483 EO units is 40148.8 Da.)

Characterization of PEGylated Variants

The PEGylation reaction may generate variants with modifications at undesired sites or result in the formation of multi-pegylated species. It is critical to localize and ensure the correct site of PEGylation. To achieve this, we performed MS analysis with a combination of in-source fragmentation (ISF) and higher-energy collision dissociation (HCD-MS/MS) on Glu-C digests of the pegylated cytokine. ISF generates peptide precursor ions with smaller PEG fragments, while MS/MS dissociates the peptide bonds, facilitating the localization of PEGylation sites.[1]

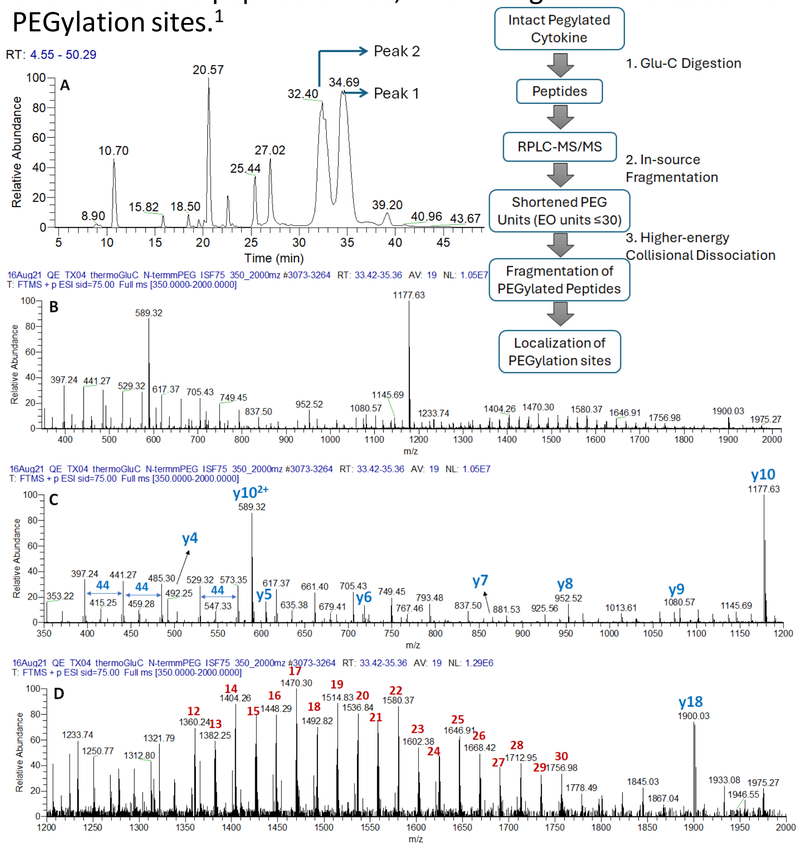


Fig. 2 (A) TIC of a Glu-C digest of a diPEGylated cytokine, **(B)** Mass spectrum after ISF of Peak 1 in Fig 2 **(C)** A zoomed-in view of *m/z* 350-1200 showing PEG fragments and peptide y-ion series. **(D)** A zoomed-in view of *m/z* 1200-2000 showing peptide precursor ions with different numbers of EO units.

Peak 1 H—OCH_2CH_2—O—CH_2CH_2CH—M[1]TP...LLK[17]CLE (n=20)

Peak 2 H—(OCH_2CH_2)—O—CH_2CH_2CH—K[35]LCATYK[41]L...L (n=20)

Fig. 2 (E) Annotated HCD-MS/MS spectrum of the peptide precursor ion with 20 EO units. **Note:** The a1 fragment ion indicates PEG attached to Met1, which also undergoes a diagnostic Met side chain loss of 48 Da (CH_3SH).

Isolation and Characterization of Oxidized Variants

Oxidation of methionine can hinder receptor binding and reduce the potency of PEGylated cytokine. Therefore, oxidation is a major product related impurity requiring careful monitoring and control throughout the drug product's manufacturing and storage process. The impact of site-specific oxidation on biological activity was assessed using H_2O_2-stressed samples. Semi-preparative HPLC was used to fractionate the individual variants followed by analysis using *in-vitro* cell-based potency assays.

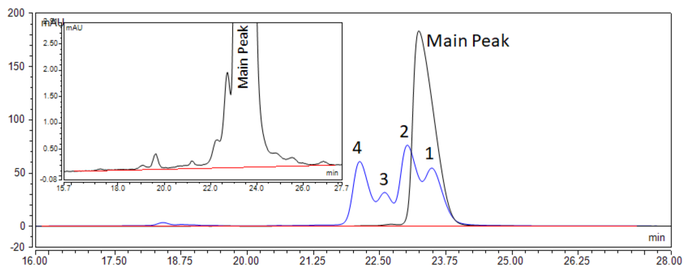


Fig. 3 Overlaid reversed phase UV chromatograms of control (black) and H_2O_2-treated (blue) PEGylated cytokine. Inset: Zoomed view.

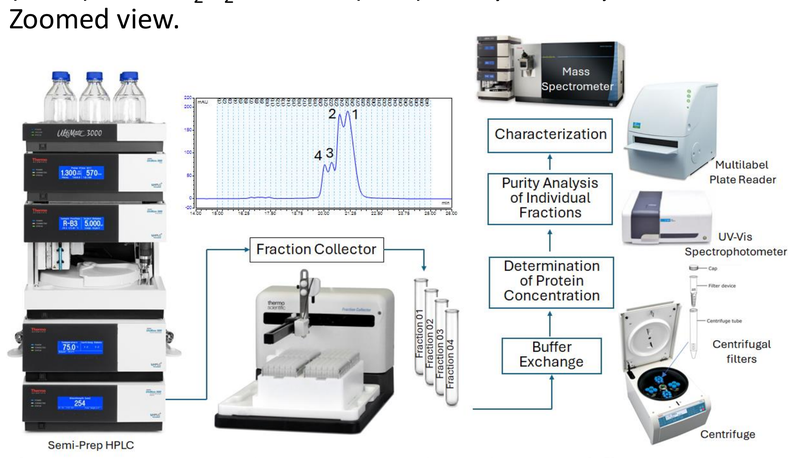


Fig. 4 Semi-Preparative HPLC Fractionation Workflow

Table 1 Characterization of oxidized variants by peptide mapping

Type of Variants	Observed MW (Da)	Δ MW (Da)	Oxidation Status	Oxidation Sites
Peak 1	40163.7	16	Single	Met[1]
Peak 2	40179.9	32	Double	Met[1], Met[138]
Peak 3	40180.1	32	Double	Met[1], Met[127]
Peak 4	40194.6	48	Triple	Met[1], Met[127], Met[138]

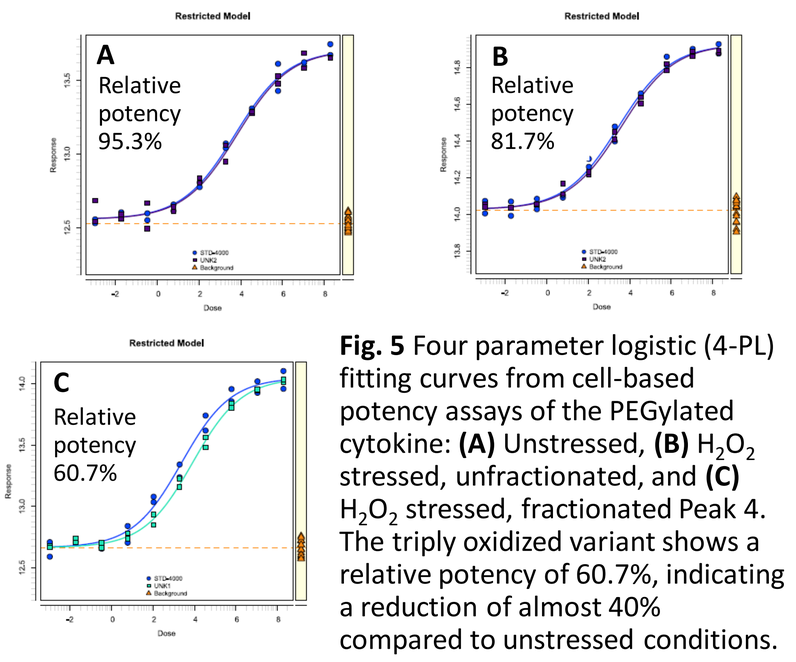


Fig. 5 Four parameter logistic (4-PL) fitting curves from cell-based potency assays of the PEGylated cytokine: **(A)** Unstressed, **(B)** H_2O_2 stressed, unfractionated, and **(C)** H_2O_2 stressed, fractionated Peak 4. The triply oxidized variant shows a relative potency of 60.7%, indicating a reduction of almost 40% compared to unstressed conditions.

Characterization of Monoclonal Antibody Variants

Recombinant monoclonal antibodies (mAbs) display structural variants, like post-translational modifications, fragmentation, dimerization, or aggregation. Common modifications include asparagine deamidation, aspartate isomerization, C-terminal lysine loss, N-terminal cyclization, among others. To characterize mAb variants, drug substance, as well as in-process and forced degraded samples, are used as sources for enriched variants. Once the variants are isolated, several advanced analytical techniques are used for comprehensive structural and functional characterization.

Table 2 Starting materials and analytical methods used for mAb product–related variant characterization

Type of Variants			Source of Enriched Variants	Analytical Methods
Size variants	HMWS	aggregate	mAb DS stressed at 65°C, 7D	CEX, SEC, SEC-MALS, icIEF, CE-SDS-LIF; Peptide mapping, disulfide mapping, and PTMs by RPLC-MS/MS, Intact mass analysis by RPLC-MS, Cell-based potency assay, and FcRn binding by SPR
		dimer (trimer)	mAb DS stressed with 0.01N NaOH, 5D	
	LMWS	LMW1	mAb DS stressed at 65°C, 7D	
		LMW2		
Charge variants	Acidic species		mAb DS stressed at 30°C for 2W in pH 4, pH 6, and pH 8 mAb DS stressed at 65°C, 2D In-process MMCF fractions	
	Basic species			
Oxidation variants			mAb DS H_2O_2 stressed	
Glycosylation variants			mAb DS/DP	Released glycan analysis by HILIC-FLD and Glycopeptide analysis by LC-MS/MS

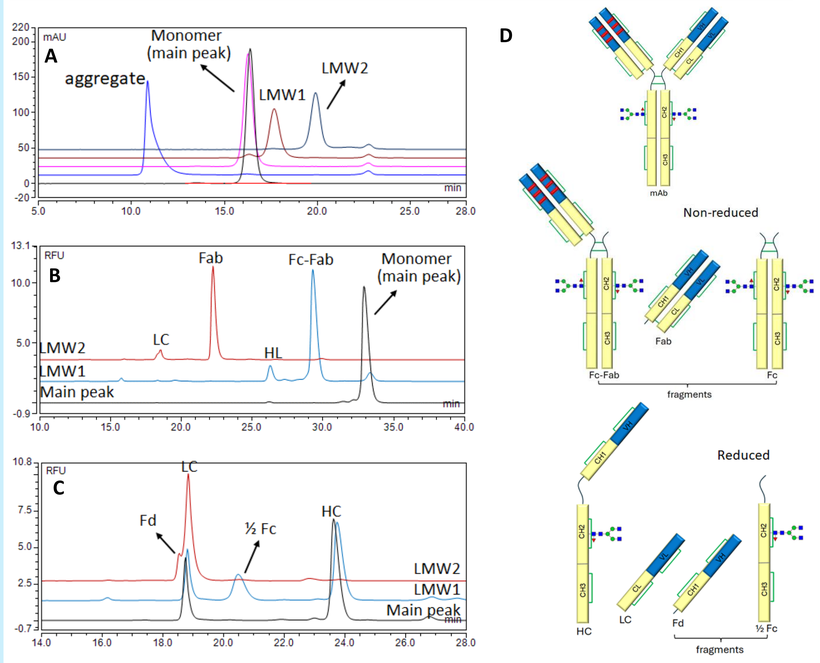


Fig. 6 (A) Overlaid SEC chromatograms of unfractionated mAb (black), and fractions of aggregate (blue), main peak (magenta), LMW1 (maroon), and LMW2 (dark blue) collected after stress at 65°C, 7D. **(B)** & **(C)** Overlaid non-reduced and reduced CE-SDS electropherograms of the monomer (main peak), LMW1, and LMW2 fractions, **(D)** Expected species following mAb hinge region fragmentation.

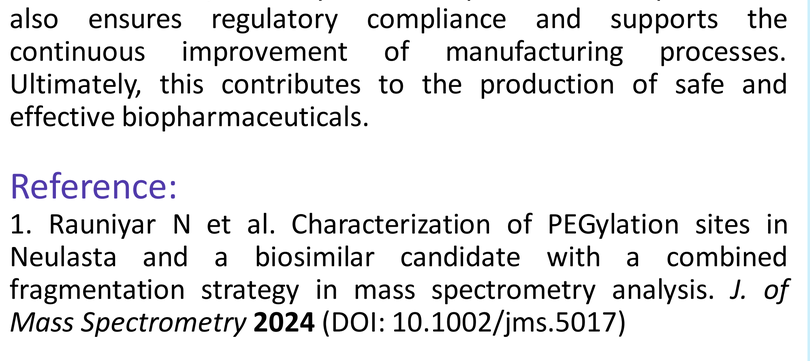


Fig. 9 CEX-HPLC chromatogram of in-house IgG1 mAb showing acidic and basic product related variants.

Conclusions

Biotherapeutics commonly exhibit multiple variants. A thorough understanding of these variants gained through characterization, not only enhances product development but also ensures regulatory compliance and supports the continuous improvement of manufacturing processes. Ultimately, this contributes to the production of safe and effective biopharmaceuticals.

Reference:

1. Rauniyar N et al. Characterization of PEGylation sites in Neulasta and a biosimilar candidate with a combined fragmentation strategy in mass spectrometry analysis. *J. of Mass Spectrometry* **2024** (DOI: 10.1002/jms.5017)