



Structural study of a PEGylated therapeutic protein by MALDI-ISD and ESI-QTOF

Structural characterization of pegvisomant using MALDI-TOF and ESI-QTOF allowed identification of four PEGylation states including a previously unpublished PEGylation state with 3 PEG moieties. Two PEGylation sites were identified by top-down sequencing utilizing MALDI-ISD in fractions containing all PEGylation states.

Introduction

Conjugation of poly(ethylene glycol) (PEG) polymer to a therapeutic protein known as PEGylation is one of the most effective methods to dramatically increase a protein's half-life in blood. Over the past 20 years, the number of PEGylated therapeutic proteins approved by the FDA has significantly increased. Only a handful of these were prepared via site-specific PEGylation while the majority were produced via non-specific PEGylation resulting in heterogeneous mixtures consisting of multiple PEGylation states and combination of different PEGylation sites for each of the PEGylation states. Each of these PEGylated conjugates may have different activity and stability properties and characterizing them is a very important bioanalytical task. Keywords: PEGylated protein, Biopharmaceutical, MALDI-TOF, ESI-QTOF, Top-down sequencing, In-source decay This study was focused on characterization of pegvisomant (Somavert[®]) using MALDI-ISD and ESI-QTOF. Pegvisomant is a protein containing 191 amino acid residues with 9 PEGylation sites including 8 lysine residues and the N-terminal α -amino group (Figure 1). According to the drug description the predominant PEGylation states are 4-6 PEG moieties per protein molecule and the PEG size is approximately 5000 Da.

Methods

A sample of a commercially available PEGylated protein pegvisomant was used for this study. Pegvisomant samples were desalted using C4 ZipTips and prepared with sinapinic acid as a matrix for intact MALDI-TOF mass measurements.

Pegvisomant (Somavert)											
FPTIP	10 LSRLF	DNAML	20 RADRL	NQLAF	30 DTYQE	FEEAY	40 I P <mark>K</mark> EQ	KYSFL	50 QNPQT	SLCFS	60 ESIPT
PSNRE	70 Etqq <mark>k</mark>	SNLEL	80 LRISL	LLI QS	90 WLEPV	QFLRS	100 UFANS	LUYGA	11.0 Sdshv	YDLL <u>K</u>	120 DLEEK
IQTLM	130 GRLED	GSPRT	140 GQIFK	QTYS <u>k</u>	150 Fdtns	HNDDA	160 LL <mark>K</mark> ny	GLLYC	170 Fnadm	SRUST	180 FLRTU
QCRSU	190 EGSCG	F									_

Figure 1: Sequence of pegvisomant (Somavert[®]) with lysine residues highlighted in green. Determined PEGylation sites (Lys158 and α -amino group) are highlighted in red

The samples were fractionated on a 2.1 x 100 mm column at 400 μ L/min flow rate using 60 min gradient then mixed with 1% solution of triethylamine pumped at 10 μ L/min flow rate prior to analysis on a maXis II UHR-QTOF instrument (Bruker Daltonics). The collected fractions were concentrated by vacuum centrifugation and prepared with sinapinic acid as a matrix for intact MALDI-TOF mass measurements. 1,5-Diaminonaphthalene (DAN) and SDHB were used as matrices for top-down sequencing using MALDI In-Source Decay (MALDI-ISD). All MALDI measurements were done on a rapifleX TOF/TOF mass spectrometer (Bruker Daltonics).



Figure 2. MALDI-TOF spectra in linear mode of pegvisomant sample before separation \land, fraction 1 🖲, fraction 2 🛈 fraction 3 🛈 and fraction 4 Ĕ



Figure 3. Portions of the deconvoluted ESI-QTOF spectrum corresponding to collected fractions for MALDI analysis. (A) Pre-collection part of the spectrum, (B) fraction 1, (C) fraction 2, (D) fraction 3 and (E) fraction 4

Results

- Intact mass measurements of pegvisomant by MALDI before separation showed PEGylation states with 4 PEG moieties at m/z 44000, 5 PEG moieties at m/z 49100 and less abundant 6 PEG moieties at m/z 54300 (Figure 2A).
- The ESI-QTOF measurements revealed a PEGylation state with 3 PEG moieties (Mr 38500) in addition to the other 3 states (Figure 3A). This PEGylation state with 3 moieties was previously unpublished.
- MALDI measurements of the four collected fractions correlated well with higher resolution ESI-QTOF measurements corresponding to the collection times (Figures 2B-2E, 3B-3E, and 4).



Figure 4. Zoomed-in region of fraction 1 from 43450 Da to 43950 Da showing resolved PEG oligomers of the PEGylated state with 4 PEG moieties



Figure 5. MALDI-ISD spectra of pegvisomant fractions that were prepared with DAN matrix. Spectrum of fraction 1 is in panel ($\hat{\mathbf{B}}$), fraction 2 in panel ($\hat{\mathbf{B}}$), fraction 3 in panel ($\hat{\mathbf{C}}$) and fraction 4 in panel ($\hat{\mathbf{D}}$)

- Fraction 1 contained predominantly the PEGylation state with 4 PEG moieties (Figures 2B and 3B). Fraction 2 primarily had the PEGylation state with 5 moieties (Figures 2C and 3C) while fraction 3 had a mixture of the states with 5 and 6 PEG moieties (Figures 2D and 3D). Fraction 4 contained the PEGylation state with 6 moieties (Figures 2E and 3E).
- The fragmentation pattern in MALDI-ISD spectra of all fractions was nearly identical and was dominated by the C-terminal y and particularly (z+2) ion series. The latter provided continuous and almost complete coverage to (z+2)33 ion corresponding to the fragmentation between Asn159 and Lys158 (Figure 5). This indicated that Lys158 is PEGylated in all fractions and all detected PEGylation states.
- The lack of N-terminal fragmentation indicated that the N-terminal α-amino group is PEGylated in all fractions (Figure 5).

Summary

- The MALDI-TOF and ESI-QTOF measurements allowed identification of four PEGylation states of pegvisomant.
- The PEGylation states with 4, 5 and 6 PEG moieties were identified by both techniques. In addition, the previously unpublished PEGylation state with 3 PEG moieties was detected by ESI-QTOF.
- Two PEGylation sites (Lys158 and α-amino group) were identified by MALDI-ISD in fractions containing all PEGylation states.

Conclusions

- Chracterization of PEGylation states and PEGylation sites is one of the most important tasks in the analysis of nonspecific PEGylated proteins
- MALDI-TOF and ESI-QTOF provide complimentary information about the PEGylation states of PEGylated therapeutic proteins.
- MALDI-ISD is helpful in the identification of PEGylation sites that are close to the N- and C-termini.





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