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

ASMS 2019, MP 668

Sergei Dikler and Anjali Alving

Bruker Scientific, LLC, Billerica, MA

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 dramatically 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. 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)

```
        10         20         30         40         50         60
FPTIP LSRLF DNAML RADRL NQLAF DIYQE FEEAY IPKEQ KYSFL QNPQT SLCFS ESIPT
        70         80         90        100        110        120
PSNRE ETQQK SNLEL LRISL LLIQS WLEPV QFLRS VFANS LVYGA SDSNV YDLLK DLEEK
       130        140        150        160        170        180
IQTLM GRLED GSPRT GQIFK QTYSK FDTNS HNDDA LLKNY GLLYC FNKDM SRVST FLRIV
       190
QCRSV 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 µL/min flow rate using 60 min gradient then mixed with 1% solution of triethylamine pumped at 10 µL/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).

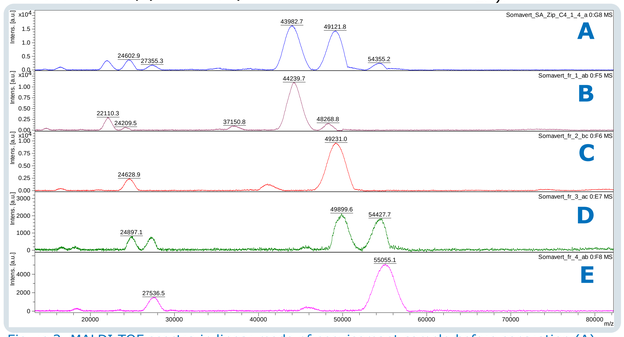

Figure 2. MALDI-TOF spectra in linear mode of pegvisomant sample before separation (A), fraction 1 (B), fraction 2 (C), fraction 3 (D) and fraction 4 (E)

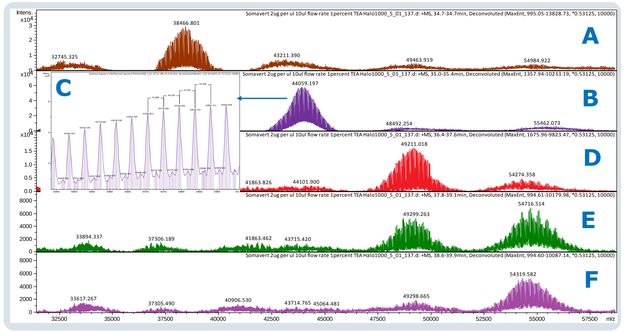

Figure 3. Portions of the deconvoluted ESI-QTOF spectrum corresponding to collected fractions for MALDI analysis. (A) Pre-collection part of the spectrum, (B) fraction 1, (C) zoomed-in region of fraction 1 from 43450 Da to 43950 Da showing resolved PEG oligomers of the PEGylated state with 4 PEG moieties, (D) fraction 2, (E) fraction 3 and (F) fraction 4

All MALDI measurements were done on a rapifleX TOF/TOF mass spectrometer (Bruker Daltonics).

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 and 3B-3F).
- 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 3D) while fraction 3 had a mixture of the states with 5 and 6 PEG moieties (Figures 2D and 3E). Fraction 4 contained the PEGylation state with 6 moieties (Figures 2E and 3F).

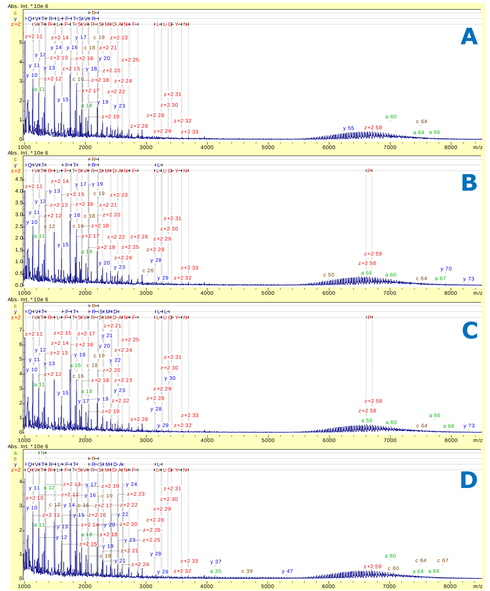

Figure 4. MALDI-ISD spectra of pegvisomant fractions that were prepared with DAN matrix. Spectrum of fraction 1 is in panel (A), fraction 2 in panel (B), fraction 3 in panel (C) and fraction 4 in panel (D)

- 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 4). 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 4).

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

- Characterization of PEGylation states and PEGylation sites is one of the most important tasks in the analysis of non-specific 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.

MALDI-TOF and ESI-QTOF

For research use only. Not for use in Clinical diagnostic procedures.