

## Direct mass spectrometric characterization of a PEGylated peptide drug using neofleX™ benchtop MALDI-TOF/TOF

neofleX benchtop MALDI-TOF/TOF offers unique capabilities for simple and fast characterization of peptide PEGylation products, thereby reducing turnaround time in biopharma development

### Abstract

We describe here a simple and fast method for comprehensive characterization of PEGylated peptide drugs using the neofleX benchtop MALDI-TOF/TOF. The method benefits from outstanding instrument performance for intact-mass measurement of peptide PEGylation products as well as neofleX's unique capabilities in top-down sequencing based on MALDI-MSD for in-situ verification of the conjugated peptide moiety.

A pre-fractionated PEGylated peptide sample comprising a 40 kDa PEG polymer and a 1900 Da peptide moiety served as an example for demonstration here. The neofleX MALDI-TOF/TOF method delivered rapid and reliable information regarding molecular weight and polydispersity of the polymer moiety as well as the polymer/peptide conjugation ratio present in the peptide PEGylation product. Instant sequence verification of the conjugated peptide moiety was achieved by MALDI-MSD-TOF and -TOF/TOF analysis, including confirmation of modification status and PEG conjugation site.

The neofleX method described here offers a highly efficient alternative to LC-ESI based analysis workflows taking particular advantage of ease of use, minimal amount of data generated, and straightforwardness of data analysis and interpretation, altogether resulting in reduced turnaround time in the development of PEGylated peptide drugs.

Keywords:  
neofleX, Polytools, Biotools,  
PEG, PEGylation, PEGylated  
peptide, polymer, peptide,  
drug, pharma, biopharma,  
MALDI-TOF, MALDI-TOF/TOF,  
high-mass analysis,  
MALDI-TDS, MALDI-MSD,  
top-down sequencing,  
T<sup>3</sup>-Sequencing

## Introduction

PEGylation, the covalent attachment of polyethylene glycol (PEG) chains to a therapeutic peptide, has become one of the most impactful chemical strategies to improve the pharmaceutical performance of peptide therapeutics by increasing in vivo half-life, enhancing solubility, and reducing immunogenicity/toxicity.

For comprehensive characterization of PEGylated peptide drugs, analytical methods are required that provide detailed information regarding both PEG and peptide moiety. MALDI-TOF/TOF mass spectrometry appears to be particularly well suited for this task because of various of its key capabilities, these are:

- Ease of use
- Robust and reliable intact-mass measurement delivering precise molecular weight and unbiased chain length distribution for synthetic polymers and polymer-peptide conjugates throughout a wide mass range
- MS/MS sequencing of peptides and proteins in both top-down and bottom-up fashion
- Minimal amount of data generated
- Rapid and simple data analysis because of few, non-overlapping MALDI charge states eliminating the need for demanding and potentially error-prone data processing pipelines commonly required in LC-ESI workflows.

We describe here a novel methodology for direct mass spectrometric characterization of PEGylated peptide drugs using the Bruker neoflex benchtop MALDI-TOF/TOF, an instrument providing research-grade performance in a lab-space-efficient form factor. In this study, pre-separated fractions of a PEGylated peptide sample comprising a 40 kDa PEG moiety and a 1900 Da peptide moiety served as an example for demonstration. The new method benefits from outstanding instrument performance in high-mass analysis allowing for simple and fast verification of the intact PEGylation product as well as unique MALDI top-down sequencing capabilities relying on MALDI in-source decay (MALDI-ISD) for in-situ verification of the conjugated peptide sequence, including modifications and conjugation site.

Overall, the MALDI-TOF/TOF method described here allows for rapid-turnaround analysis of PEGylated peptide drugs enabling instant decision-making in biopharmaceutical development.

## Experimental

All PEG polymer and PEGylated peptide samples were donated by an external party. The PEG educt polymer had an expected molecular weight of approximately 40 kDa featuring succinimidyl carbonate (SC) as alpha and omega end groups.

The  $\alpha,\omega$ -SC-PEG was conjugated with a 1900 Da peptide via the peptide's C-terminal lysine residue. The peptide moiety comprised 15 amino acid residues and carried various modifications, such as disulfide bridge, N-terminal acetylation and tryptophane methylation.

The PEGylated peptide samples represented three fractions collected from a preparative separation of the PEGylation product described above (details of separation method not disclosed).

The PEG educt polymer was dissolved in tetrahydrofuran (THF) at 10 mg/mL concentration. The PEGylated peptide samples were dissolved in 50/50 (v/v) methanol / TFA 0.1% in water at 10 mg/ml.

All samples were prepared on a Bruker MTP 384 Ground Steel MALDI target plate applying default Bruker MALDI matrix preparation protocols. The following MALDI matrices were used:

- DCTB with NaTFA as a cationizing agent (for intact-mass analysis of the PEG educt polymer)
- 2,5-Dihydroxyacetophenone (DHAP) with diammonium citrate as an additive (for intact-mass analysis of the PEGylated peptide samples)
- Sinapinic acid (SA) for verification of peptide identity by top-down sequencing (MALDI-ISD) under non-reducing conditions
- 1,5-Diaminonaphthalene (DAN) for verification of peptide identity by top-down sequencing (MALDI-ISD) under reducing conditions

All data were acquired on a Bruker neoflex benchtop MALDI-TOF/TOF instrument in positive ion polarity mode. For intact-mass analysis of the PEG polymer and PEGylated peptide samples, the instrument was operated in linear MALDI-TOF mode. In-situ verification of the peptide moiety by top-down sequencing was performed in MALDI-ISD-reflectorTOF and -TOF/TOF mode, respectively.

For external  $m/z$  calibration, the following reference standards were used:

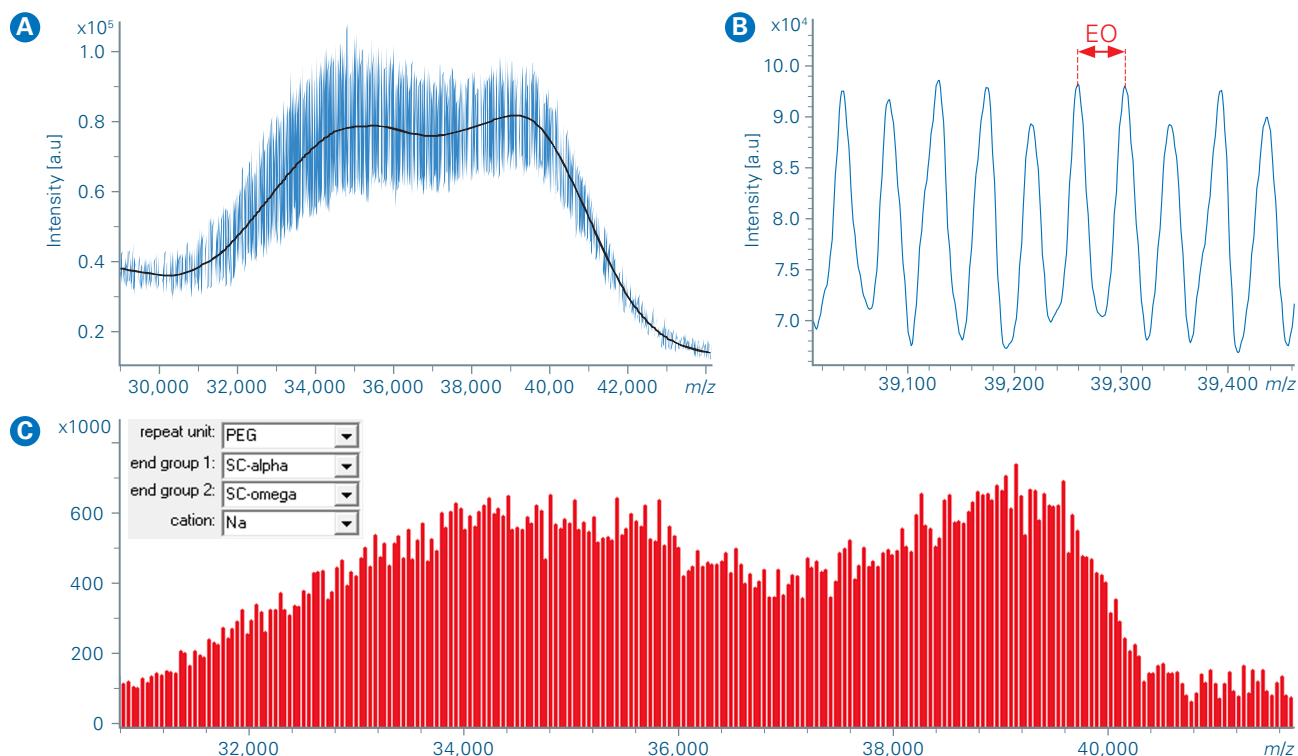
- 40 kDa Polymethylmethacrylate (PMMA)
- 29 kDa Carbonic Anhydrase isoenzyme II (*bos taurus*)
- Bruker Protein Calibration Standard
- Bruker Peptide Calibration Standard II

Data were post-processed (smoothing, baseline correction, peakfinding) in Bruker flexAnalysis software.

Data interpretation was performed using Bruker Polytools and Biotools software, respectively.

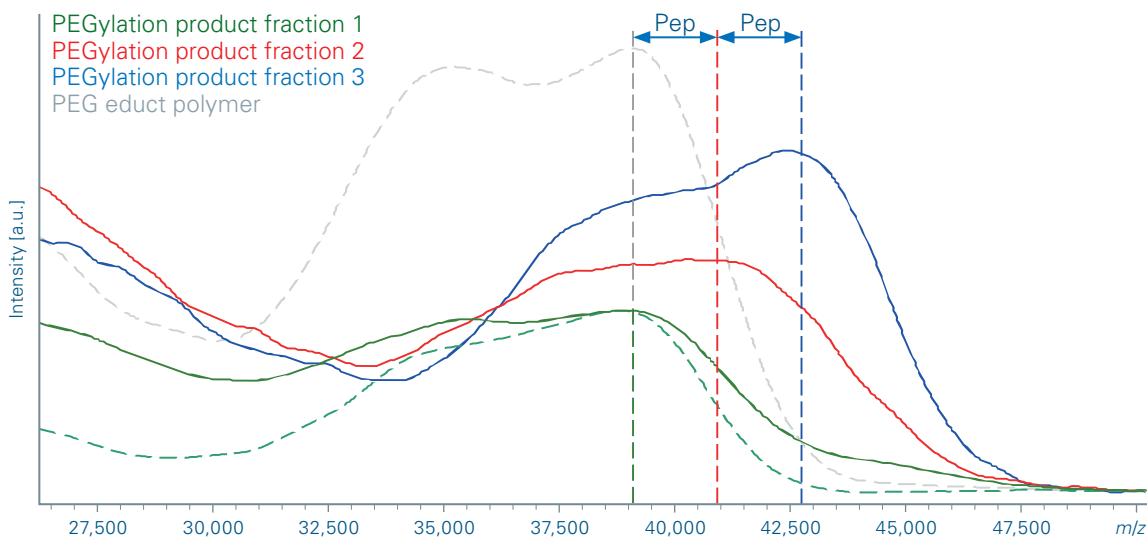
## Results

### Intact-mass analysis of the PEG educt polymer and the pre-fractionated peptide PEGylation products



**Figure 1.** Intact-mass MALDI-TOF spectrum of the 40 kDa PEG educt polymer acquired in linear mode.

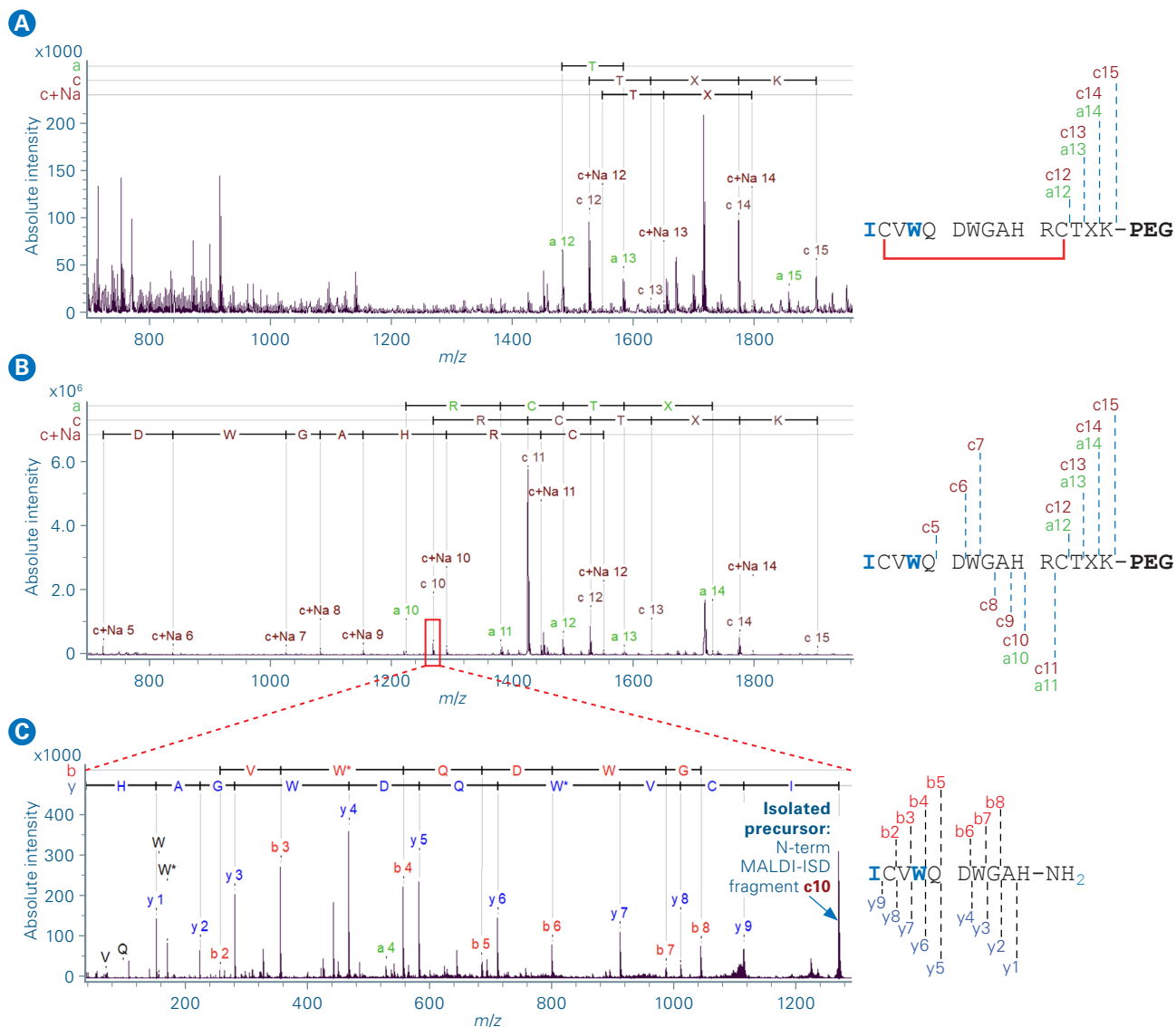
The overview spectrum (A) shows the PEG chain length distribution ranging from 30 to 44 kDa approximately. The blue colored trace represents the raw spectrum without smoothing, the black line represents the same spectrum after smoothing. A 44 Da distance between adjacent signals in the raw MALDI-TOF spectrum (B) confirms the polymer repeat unit ethylene oxide (EO). Spectrum annotation in Bruker Polytools software (C) suggests the vastly dominating presence of a single PEG distribution featuring two intensity maxima. Analysis of a 40 kDa polymer reference standard under identical experimental conditions (data not shown) delivered a Gaussian-shaped distribution proving the absence of artificial in-source fragmentation and, thus, confirming the bimodal distribution as a characteristic feature of the PEG educt polymer.



**Figure 2.** Intact-mass MALDI-TOF spectra of the pre-fractionated PEGylated peptide samples acquired in linear mode.

For comparison, the spectrum of the PEG educt polymer sample is displayed in grey color in the background. Dashed-line spectra were acquired using DCTB/Na<sup>+</sup> matrix, solid-line spectra were acquired using 2,5-DHAP matrix. The mass spectra of PEGylation product fraction 1, irrespective of the MALDI matrix in use, show a mass distribution consistent with the one detected in the spectrum of the PEG educt polymer suggesting the PEG polymer without a conjugated peptide moiety being the major compound in fraction 1. The mass spectra of PEGylation product fractions 2 and 3 show incremental shifts of the mass distribution by 1790 Da approximately reflecting the conjugation of 1 and 2 peptide molecules per PEG molecule, respectively. Accordingly, the data suggest the PEGylated mono-peptide as major compound in fraction 2 and the PEGylated di-peptide as major compound in fraction 3.

### Sequence verification of the conjugated peptide moiety by MALDI top-down sequencing



**Figure 3. Sequence verification of the conjugated peptide by MALDI top-down sequencing.**

Amino acid single-letter code X stands for 2-[2-(2-aminoethoxy)ethoxy] acetic acid (AEEA). Modified amino acids (acetylated I1 and methylated W4) are indicated on the sequence in bold blue. **(A)** MALDI-MSD-reflectorTOF spectrum of PEGylation product fraction 3 (PEGylated dipeptide) acquired under non-reducing conditions (MALDI matrix SA). In-source decay of the peptide backbone yielded a series of N-terminal a- and c-type fragments covering peptide sequence range [12-15]. Almost complete absence of fragment ions originating from peptide sequence range [1-11] confirms the presence of a disulfide bridge crosslinking cysteine residues 2 and 12. **(B)** MALDI-MSD-reflectorTOF spectrum of PEGylation product fraction 3 (PEGylated dipeptide) acquired under reducing conditions (MALDI matrix 1,5-DAN acting as a reducing agent). An extended series of N-terminal a- and c-type MALDI-MSD fragment ions confirms the sequence of the reduced peptide in the range [5-15]. Peptide sequence region [1-4] remains unconfirmed as the corresponding MALDI-MSD fragments fall outside the  $m/z$  range recorded in the MALDI-MSD-TOF spectrum. **(C)** Isolation and TOF/TOF fragmentation (i.e. T<sup>3</sup>-Sequencing) of N-terminal MALDI-MSD fragment **c<sub>10</sub>** ( $m/z$  1269.58) allowed for gapless confirmation of peptide sequence range [1-10]. Altogether, MALDI-MSD-TOF and -TOF/TOF data provided unambiguous confirmation of the conjugated peptide at 100% MS/MS sequence coverage including successful verification of its modification status and the C-terminal lysine residue as PEG conjugation site.

## Conclusion

- neoflex benchtop MALDI-TOF/TOF provides a highly capable platform for direct mass spectrometric characterization of PEGylated peptide drugs.
- Intact-mass MALDI-TOF analysis of peptide PEGylation products benefits from neoflex's exceptional high-mass performance yielding rapid and reliable information regarding molecular weight and polydispersity of the polymer moiety as well as the polymer/peptide conjugation ratio.
- neoflex's unique MALDI top-down sequencing capabilities enable instant sequence verification of the conjugated peptide moiety by MALDI-ISD-TOF and -TOF/TOF analysis, including confirmation of modification status and PEG conjugation site.
- The neoflex MALDI-TOF/TOF method described here represents a simple and fast alternative to LC-MS based analysis workflows taking particular advantage of ease of use, minimal amount of data generated, and straightforwardness of data analysis and interpretation, altogether contributing to significantly reduced turnaround time in the development of PEGylated peptide drugs.

For Research Use Only. Not for use in clinical diagnostic procedures.

### **Bruker Switzerland AG**

Fällanden · Switzerland  
Phone +41 44 825 91 11

### **Bruker Scientific LLC**

Billerica, MA · USA  
Phone +1 (978) 663-3660

