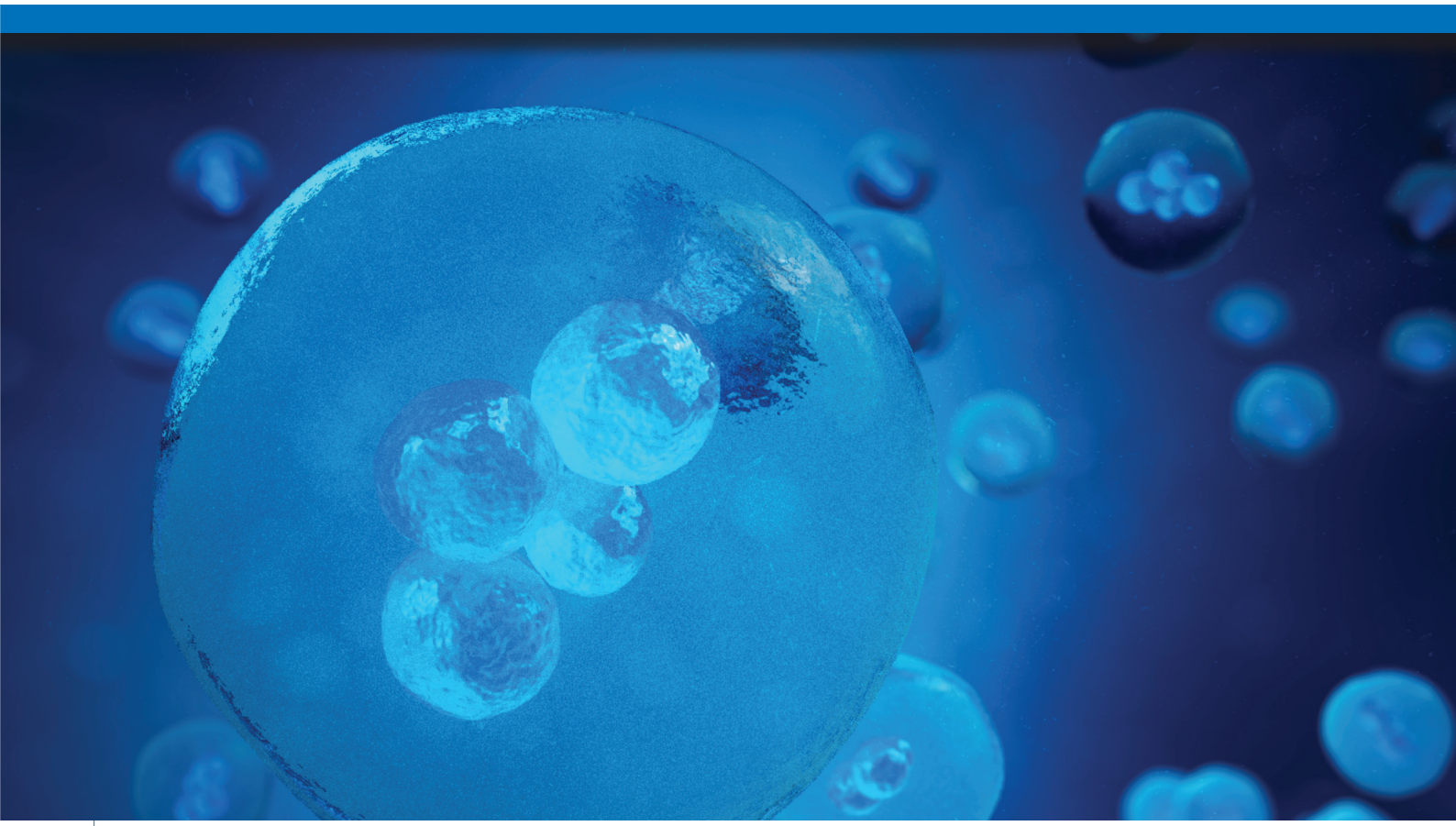

Characterization of a fusion protein under native and denaturing conditions with maXis II

Abstract

The maXis II UHR-QTOF was used to characterize key attributes of a fusion protein in its native conformation as well as under denaturing conditions. The maXis II high resolving power enabled the detection of the various glycoforms on the protein monomer and confirmed that the same glycosylation pattern was present on the fusion protein dimer. Additionally a sequence variant expressed by only one of two clones was detected by the denaturing method despite a mass difference of only 8 Da.

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Introduction

Fusion proteins, the genetic combination of two or more originally separate proteins, are highly successful biopharmaceuticals and can offer a combination of attributes that enhance their ability to treat disease. They are often used

Keywords: maXis II, Fusion protein, Glycosylation, Data Analysis software, Dissect

in the context of bio-betters, providing improved pharmacokinetic and pharmacodynamic values to the original therapeutic.

One challenge encountered when developing a fusion protein can be additional molecular complexity. While glycosylation is usually straightforward to assess on a recombinant antibody, fusion proteins may contain a large number of N- and O-glycosylation sites. This creates a significant challenge for intact mass analysis of the protein by electrospray ionization, as the potentially hundreds to thousands of glycoforms present are further convoluted by the multiple charge states of ions created. A high resolution mass analyzer is therefore essential to successfully generate a deconvoluted spectrum for such proteins.

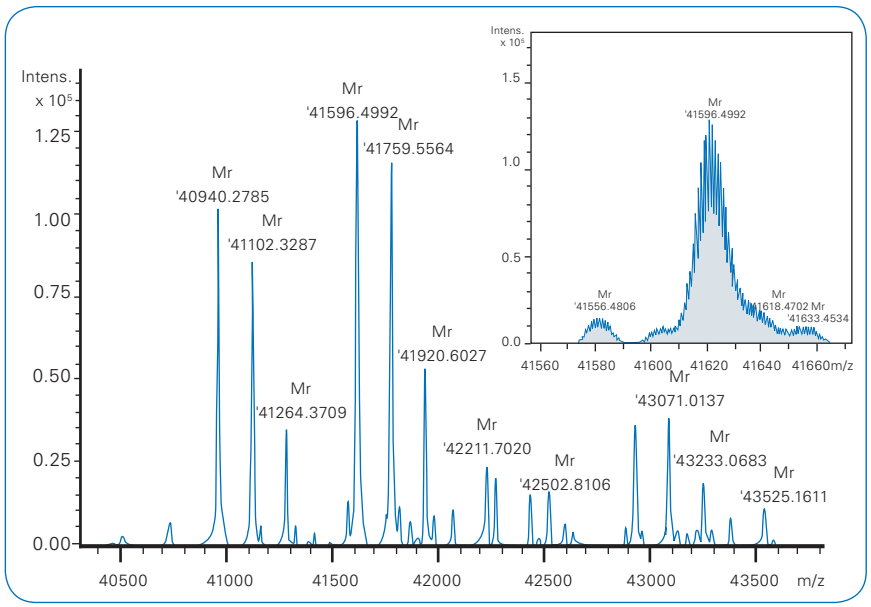

Figure 1: Fusion protein monomer - Maximum Entropy Deconvolution and SNAP II peak picking shows monoisotopic mass

Here we utilize the maXis II UHR ESI QTOF mass spectrometer (Bruker Daltonics, Billerica, MA) to characterize a fusion protein in denaturing and native conditions to determine the glycosylation mass profile of its monomer and non-covalent dimer forms, respectively. Additionally the high resolution

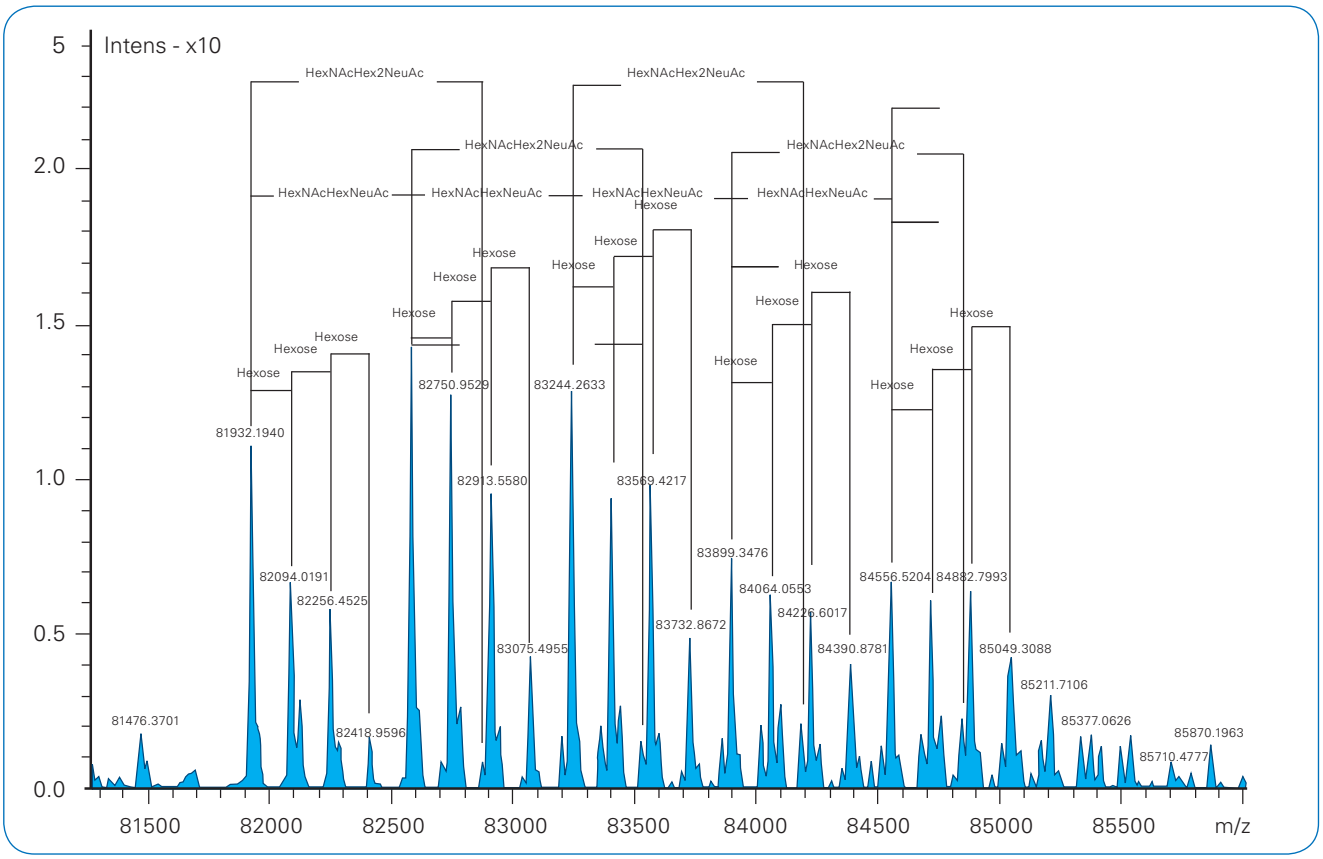

Figure 2: Fusion Protein Dimer by Native Spray MS – Confirmation of expected glycosylation pattern

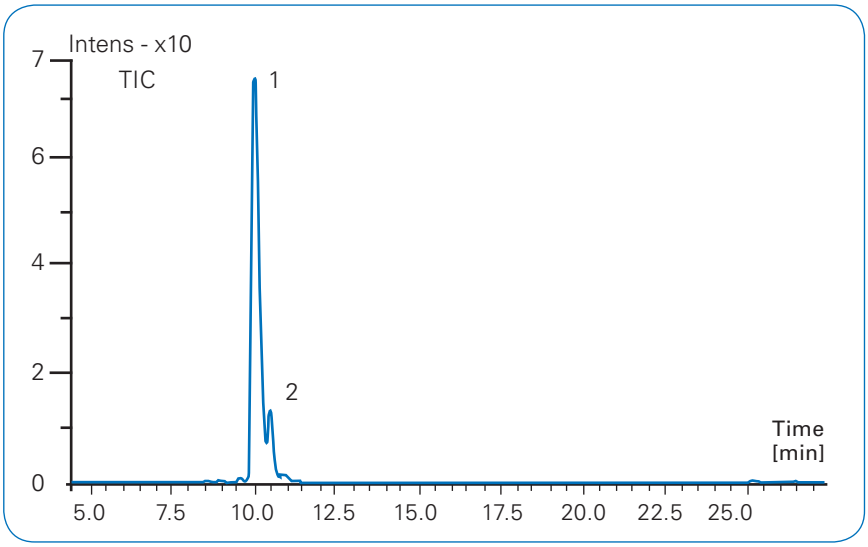

Figure 3: Separation of a sequence variant of the fusion protein seen as peak # 2

of the maXis II enabled the detection of a sequence variant of 8 Da on a second fusion protein produced from one of two different clones. The dissect command in Data Analysis™ software (Bruker Daltonics, Billerica, MA) was used to automatically find compounds on an LC-MS chromatogram trace; this algorithm is based on the principle that all ions that have the maximum intensity at the same time belong to the same compound, thus making it possible to find distinct compounds even if the peaks almost completely overlap.

Experimental

Test Materials and Instrumentation

Samples from two fusion protein bio-therapeutic programs (here titled BMS Protein 1 and BMS Protein 2) were provided by Bristol-Myers Squibb (Hopewell, NJ). Samples of BMS Protein 1 were evaluated using native spray analysis, and BMS Protein 2 for sequence variant determination. The workflow included the maXis II UHR-QTOF and Data Analysis software (Bruker Daltonics, Billerica, MA), and UltimateTM 3000 RSLC (Thermo Scientific, San Jose, CA).

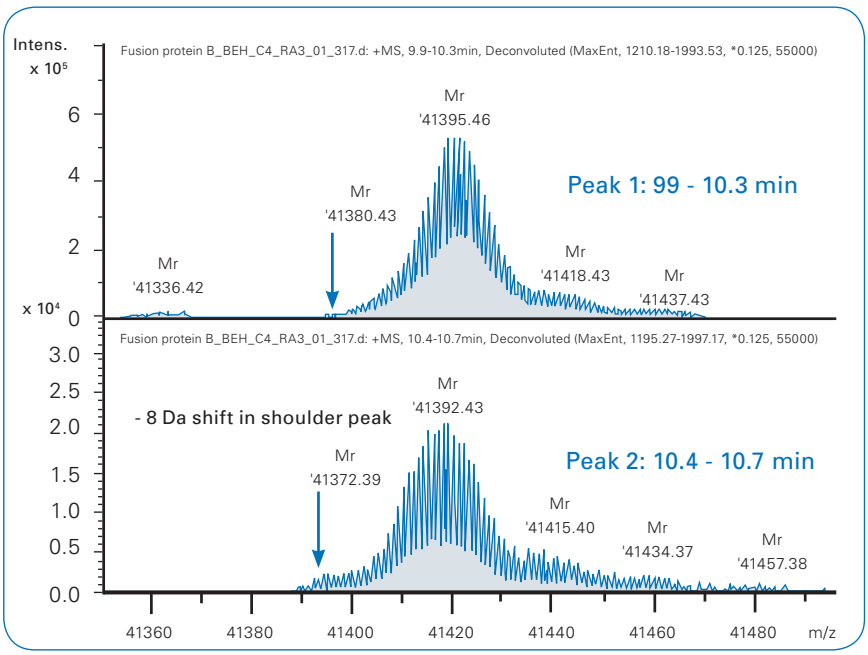

Figure 4: Mass spectra of the mutant protein in peak #2 showing a -8 Da mass shift

Results and Discussion

LC-MS of Fusion Protein Monomer in Denaturing Conditions

The non-covalent dimer of the fusion protein BMS Protein 1 was first analyzed under denaturing LC-MS conditions with an Acquity UPLC BEH C4, 2.1 x 100 mm column (Waters, Milford, MA). The mobile phases were water + 0.1 % formic acid + 0.05 % TFA and acetonitrile + 0.1 % formic acid + 0.05 % TFA with a 30 min gradient at a flow rate of 0.3 ml/min. The high resolving power of the maXis II enabled the isotopic resolution of the fusion protein while maintaining the True Isotopic Pattern™ (TIP) of isotope spacing and intensity under fast HPLC conditions. This enables protein modifications to be easily determined and accurately quantified. The SNAP II peak picking algorithm was used to determine the monoisotopic mass of the fusion protein glycoforms with high mass accuracy (Figure 1).

The observed delta-masses on the fusion protein mass spectrum were consistent with the expected predominant O-glycoforms, as shown in Table 1.

LC-MS of Fusion Protein Dimer in Native Conditions

Analysis of the non-covalent fusion protein dimer BMS Protein 1 was carried out under native conditions. After

The **Compounds - Dissect** command is used to automatically find compounds on an LC-MS chromatogram trace even if the peaks almost completely overlap

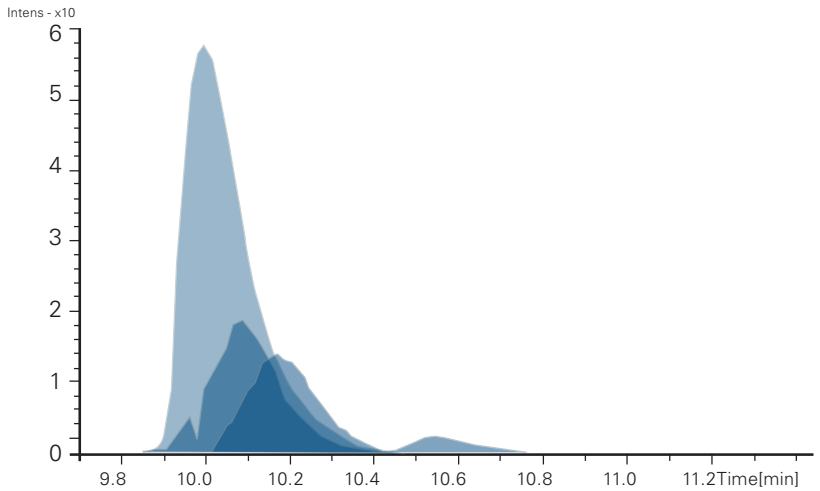

Figure 5: Dissect detection of co-eluting compounds

a buffer exchange into 10 mM ammonium bicarbonate using a molecular weight cut off (30 kDa) spin filter, 15.6 µg of the fusion protein was loaded on a Polyhydroxyethyl A 3 µm, 1 x 50 mm column (PolyLC Inc., Columbia, MD) and eluted under isocratic conditions with 100 mM ammonium at a flow rate of 15 µl/min for 10 min. Native Spray of the intact fusion protein allowed the visualization of the non-covalent fusion protein dimer (Figure 2). The glycosylation pattern was consistent with the glycans observed on the monomer of the fusion protein (Figure 1).

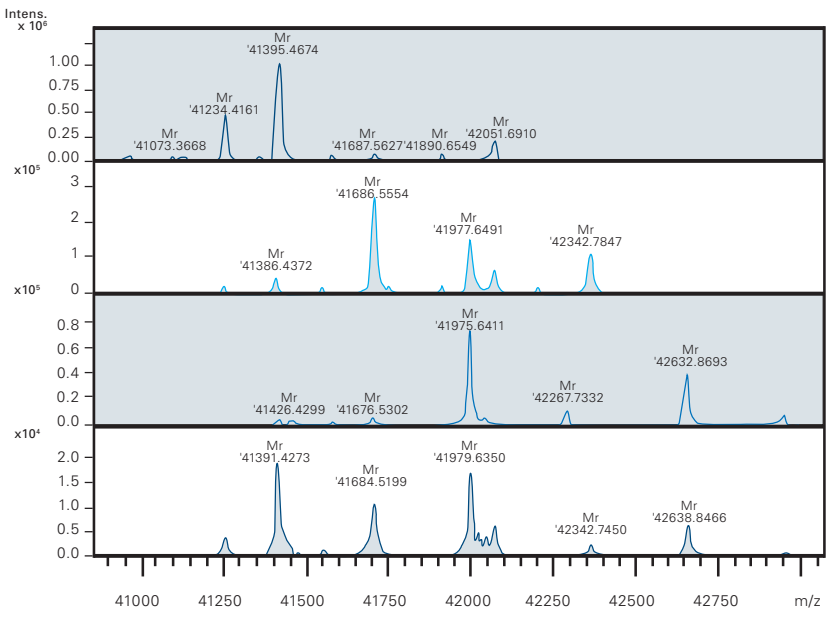

Figure 6: Deconvoluted mass spectra of Dissect detected compounds

Sequence Variant Determination

A second fusion protein, BMS Protein 2, was expressed from a clone containing a sequence variant with an abundance of ~20%. This variant corresponded to a mass shift of 8 Da. In order to detect the sequence variant, the fusion protein samples were analyzed by LC-MS under denaturing conditions as described above, and the resulting spectra were analyzed using Data Analysis. Figure 3 shows the chromatographic separation of a sequence variant (peak # 1) from the expected protein (peak # 2). An examination of the mass spectra corresponding to the two chromatographic peaks revealed a -8 Da mass shift in the small peak # 2 (Figure 4).

Data Analysis™ Software and Dissect

Figure 5 illustrates the Dissect algorithm that is used for compound detection in an LC-MS chromatogram even if the peaks completely overlap. Four compounds were identified by the Dissect software and their corresponding mass spectra seen in Figure 6 shows distinctly different species which include spectra under the main peak and lower intensity heterogeneities eluting under the overlapping compounds.

The Dissect algorithm utilizes fuzzy-logic algorithms, which allow a peak separation process to be run without the need for user interaction or any prior information. The Dissect algorithm makes it possible to separate overlapping peaks even if their retention times differ by half the scan time and their intensities differ by more than one order of magnitude.

Table 1. Expected glycosylation on fusion protein

Glycan	Monoisotopic Mass (Da)	Elemental Composition
Hexose	162.05282	$C_6H_{10}O_5$
HexNAc-Hex-NeuAc	656.22761	$C_{25}H_{40}N_2O_{18}$
HexNAc-Hex-2xNeuAc	947.32303	$C_{36}H_{57}N_3O_{26}$

Conclusions

- The maXis II UHR ESI QTOF mass spectrometer was used to obtain isotopic resolution of the 40 kDa monomer of a highly glycosylated fusion protein. The SNAP II peak picking algorithm calculated the monoisotopic molecular weight from isotopically resolved peaks with high isotopic fidelity (True Isotopic Pattern ™)
- Native spray data of the 80 kDa non-covalent fusion protein dimer confirmed the expected glycosylation pattern
- The maXis II UHR ESI QTOF mass spectrometer was also able to detect a sequence variant corresponding to a -8 Da mass shift that was present at ~20% abundance.
- The Dissect software enabled identification of 4 distinctly different species despite overlapping peaks in the chromatogram.

Acknowledgments

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