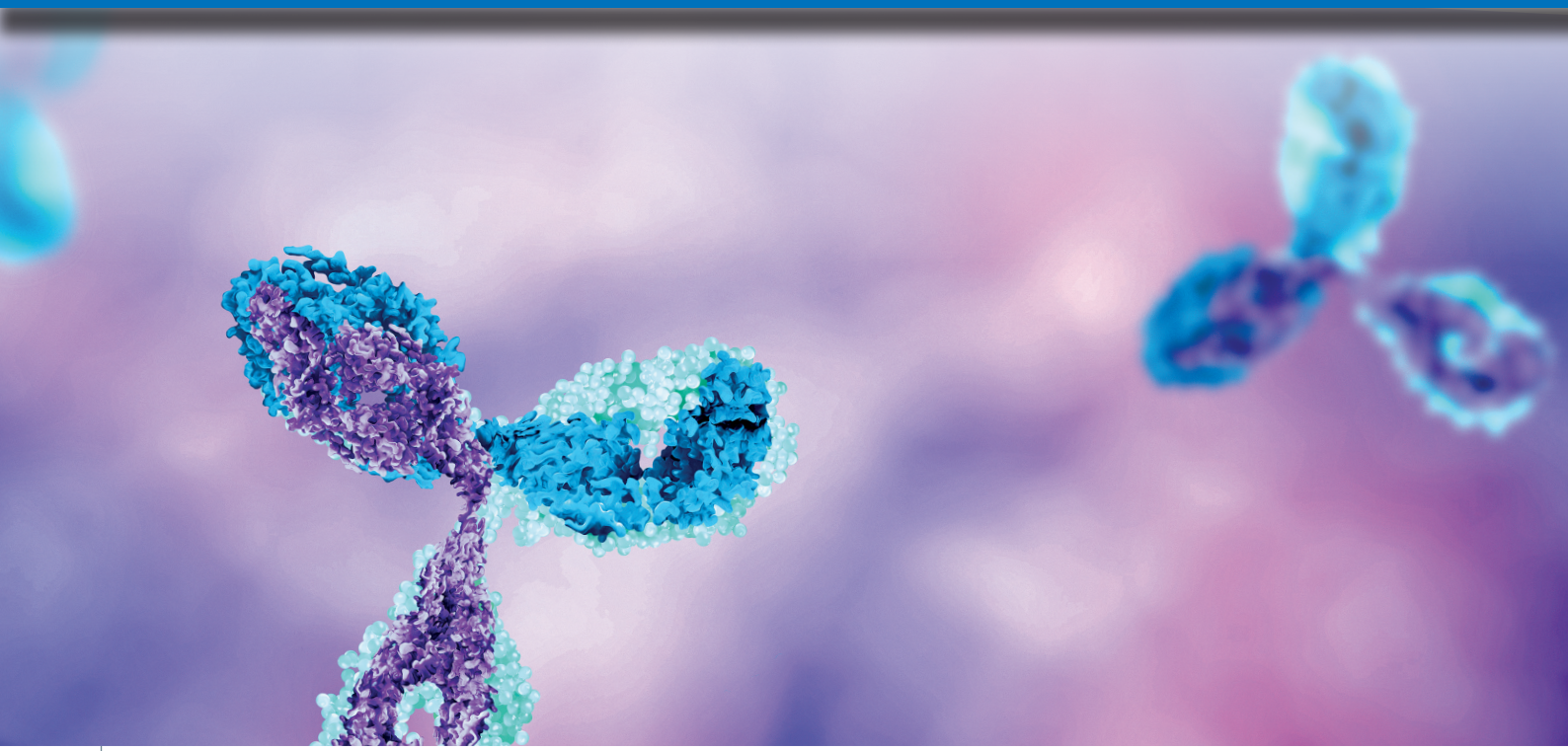

Extensive LC-Top-Down MS Sequence Confirmation of the NISTmAb Reference Material 8671

Characterization of therapeutic antibodies aims on primary sequence validation, localization of modifications such as glycan profiles and the determination of disulfide bond status of the target molecule.

Introduction

Typically, LC-ESI-MS/MS bottom-up analyses with multiple enzyme digests are used for sequence confirmation. A much faster approach to confirm suspected sequences relies on intact mass determinations or – in case of monoclonal antibodies – on middle-up analysis: the cleavage near the hinge region of the heavy chain (HC) using enzymes like IdeS followed by reduction. The method for protein sequence analysis introduces a very low level of chemical artefacts and yields the Fd, Fc/2 and LC domains in the 23-27 kDa mass range, which can be determined with a Bruker maXis reliably with 1 ppm accuracy. However, the direct sequence analysis of these large protein fragments still remains a challenge using electrospray instrumentation due to the complexity of ETD spectra.

Here MALDI-TOF Top-Down Sequencing (TDS) was utilized as an orthogonal method to provide extensive sequence information from NISTmAb IdeS fragments

Keywords:
spotOn, inert gas mass option, nanoElute, rapifleX TOF/TOF, BioPharma Compass 3.0, MTP AnchorChip 384

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to validate large fractions of each sequence and at the same time provide reliable information about the N- and C-terminal modification and truncation status, respectively.

LC-MALDI-TOF setup

Top-Down LC-MALDI workflows are now as easy to run as LC-ESI with the new **spotOn** fraction collector (Figure 1) but provide a uniquely high level of sequence confirmation and assessment of N- and C-terminal modification status. LC-fractions containing, e.g., the Fd, Fc/2 and LC domains, are automatically collected and prepared on Bruker AnchorChip sample plates with MALDI matrix without any further manual user intervention delivering ready-to-go MALDI sample plates which can be subjected to MALDI-TOF data acquisition.

Top-Down data quality increases if technical artefacts such as oxidation can be prevented. To protect protein samples from oxidation, an inert gas mask can be applied and flooded, e.g., with nitrogen during fraction deposition and solvent drying.

Methods

The IgG1 standard NISTmAb Reference Material 8671 was IdeS digested and reduced in 6 M guanidinium chloride, 100 mM DTT for 3 h at 50°C. A vial with reduced IdeS digest was placed into the autosampler of the nanoElute nano-LC system. The MALDI sample holder and 2 bottles with sDHB matrix (Bruker # 8209813) and with 10 pmol/µl ubiquitin in sDHB solution were placed onto the matrix and calibrant positions of the spotOn target tray (Bruker). Fraction collection and matrix deposition was controlled by the Hystar spotOn plug-in and the spotOn wizard was used guiding the user step-by-step through the setup to ensure best quality of target preparation ready for MALDI analysis

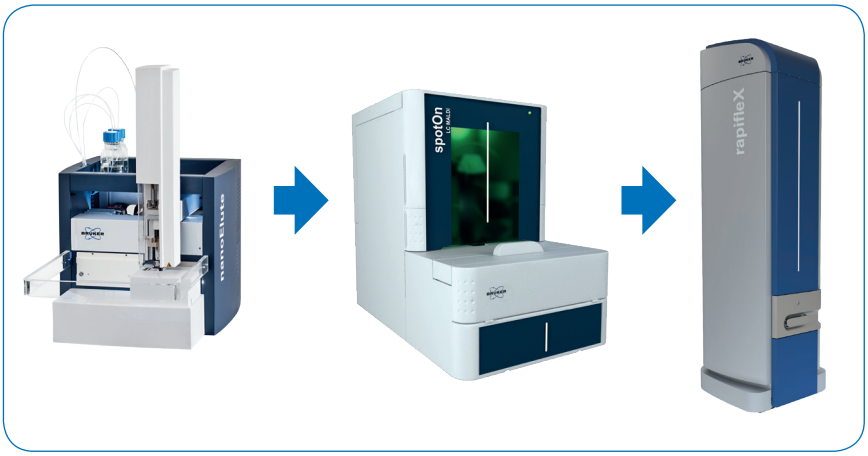

Figure 1: spotOn sample spotter (center) with nanoElute liquid chromatograph and rapifleX MALDI-TOF/TOF mass spectrometer

(Figure 2). The inert gas mask accessory (Bruker # 1855390) was used to protect the mAb subunits from oxidation providing for higher sequence coverage.

Four pmol of the reduced IdeS digest were injected and separated using the nanoElute equipped with a Fortis C_4 separation column (dichrom). A gradient generated of 60% acetonitrile

Table 1: Experimental conditions

	Experimental Details
Nano Flow UHPLC	nanoElute (Bruker)
Fraction Collector	spotOn plus inert gas mask (Bruker)
MALDI Mass Spectrometer	rapifleX-TOF/TOF (Bruker)
Software	Hystar 5.0 with nanoElute and spotOn plug-in; Compass for flexseries 2.0; BioPharma Compass 3.0 (Bruker)
Sample	NISTmAb reference material RM 8671 (NIST)
Enyme	IdeS (FabRICATOR, Genovis)
Separation Column	FortisBio C4 1.7 µm, 150 mm x 200 µm (dichrom)
Solvent A	0.1% TFA in water
Solvent B	60% acetonitrile, 40% n-propanol, 0.1% TFA
Flow rate	2.0 µl/min
Gradient	0-10 min: isocratic flow at 5% B 10-35min: linear gradient 5-55% B
MALDI Sample Plate	MTP AnchorChip 384 (Bruker)
Matrix	20 mg/ml sDHB (Bruker) in 30% acetonitrile/water/0.1% TFA
Calibrant	bovine ubiquitin (Sigma) in sDHB (Bruker)
spotOn Workflow	Cap 2
Fractions	192 fraction of 15 s each

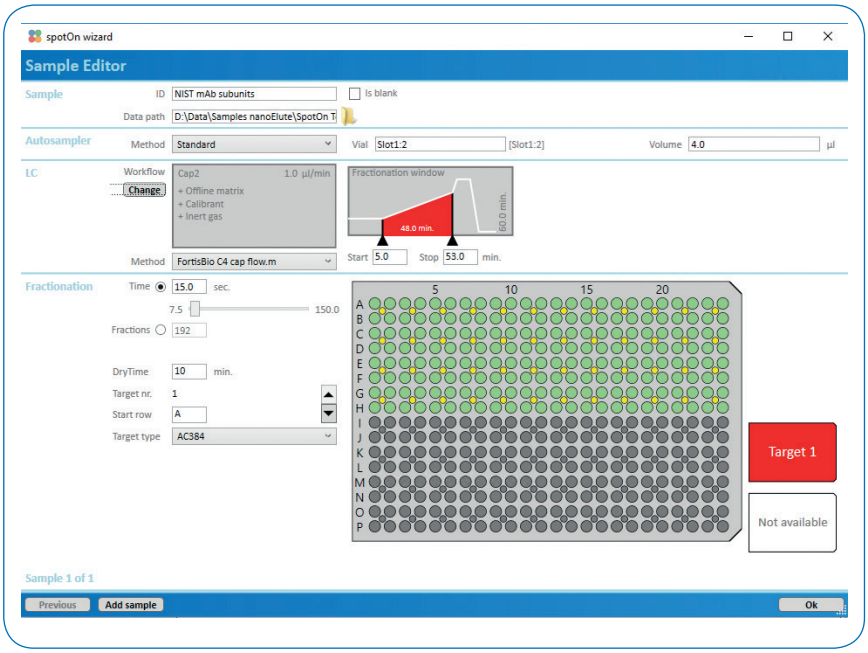

Figure 2: spotOn wizard showing the settings of separation, fraction collection (green spots) and matrix preparation including subsequent calibrant spotting (yellow spots) including the use of the inert gas mask

and 40% n-propanol with 0.1% TFA (mobile phase B) and water/0.1% TFA (mobile phase A) was used to separate the subunits. The spotOn settings were selected as predefined in a workflow for protein analysis (fraction collection and MALDI sample preparation). Fractions were collected on the 800 µm anchor spots of a MTP Anchorchip 384 BC sample plate (Bruker # 8280790) and subsequently prepared with the MALDI matrix sDHB by the spotOn. In addition, bovine ubiquitin was spotted as TDS standard on the calibration spots positions. All steps were performed under N_2 gas using the spotOn inert gas mask. The fully prepared MALDI sample plate was then inserted into a rapiflex TOF/TOF and automatically MS acquired using the run file provided by the spotOn control software (Figure 2).

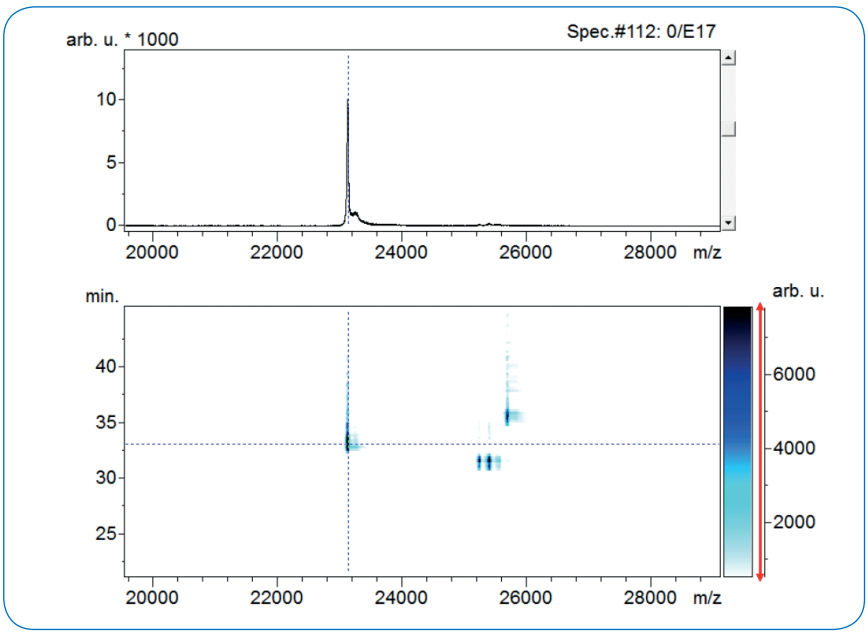

Figure 3: LC-MALDI MS analysis of IdeS subunits of NISTmAb. The Fc/2 with the glycan profile G0F, G1F and G2F was detected at Rt 31.5 min, the LC at 33.5 min and the Fd at 35.5 min. The spectrum (top panel) shows the fraction containing the LC after selection in the Survey Viewer (bottom panel)

For the automated measurement of the sDHB crystals on the AnchorChip plate, the laser raster file three_circles was selected in the AutoXecute method, which directs the laser beam to the crystal rim of the anchor spots.

Results

The reduced NISTmAb IdeS digest was LC-analyzed, spotted onto the sample plate and MALDI MS spectra were automatically acquired. The Fc/2, LC and Fd were well separated in different fractions (Figure 3) and each subunit was subsequently TDS analysed manually.

Top-down sequencing with MALDI used the in-source decay (ISD) method of the Bruker MALDI instruments (ultrafleXtreme, rapifleX) in reflector positive mode, providing for the detection of ISD fragments in the mass range from 800 to 10.000 Da or higher. Spectra were accumulated with several thousand laser shots across the rim of the respective spots on the target fraction position. MALDI-ISD spectra with peaklists obtained in flexAnalysis were analyzed in BioPharma Compass (BPC).

The sequence coverage of the NISTmAb LC in the TDS spectrum is 73.2%, the sequence validation coverage (SVP) 85.4%. [1] All three complementarity determining regions (CDRs) are covered from ISD fragments and therefore fully confirmed.

The TDS spectrum of the Fd was analyzed in BPC 3.0 matching 2 sequence variants: one carrying N-terminal pyro-glutamination and one without modification. The

N-terminus was confirmed with 75 matching residues for the pyro-glutaminated sequence while confirmation was not obtained for the non-modified sequence. The overall sequence coverage was 61%, SVP was 72%. Two of the three CDRs were covered and confirmed, too. The C-terminal sequence was confirmed while the SVP was 72% only and thus lower than the typical threshold setting of > 90%, meaning that 28% percent of the core sequence were not covered by fragments ions (Figure 5).

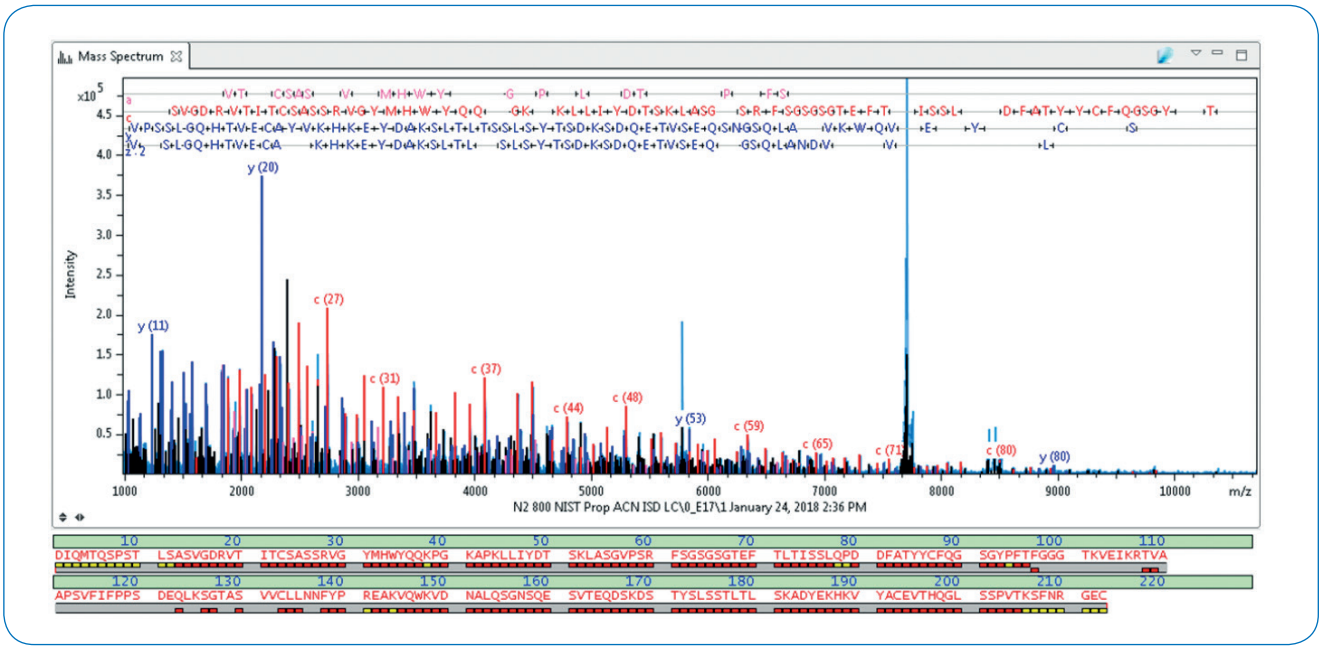

Figure 4: MALDI-TDS spectrum of the NISTmAb LC with annotion of N- and C-terminal ion series in red (c-, a.ions) and blue (y, z+2-ions), respectively. The residues covered by ISD fragments are shown in the protein sequence coverage view sa colored "bricks" (bottom). N-terminal 97 and C-terminal 77 residues were confirmed including proper terminal status

Rank	Protein	Modifications	N-term...	C-term...	Sequen...	SVP [%]	SC [%]	N-term Ma...	C-term Ma...
1	NIST Fd pGln:Fd	Gln->pyro-Glu: nterm	yes	yes	no	72.4	61.1	75 (1-75)	88 (152-239)
2	NIST Fd pGln:Fd		no	yes	no	42.3	36.0	3 (10-12)	88 (152-239)

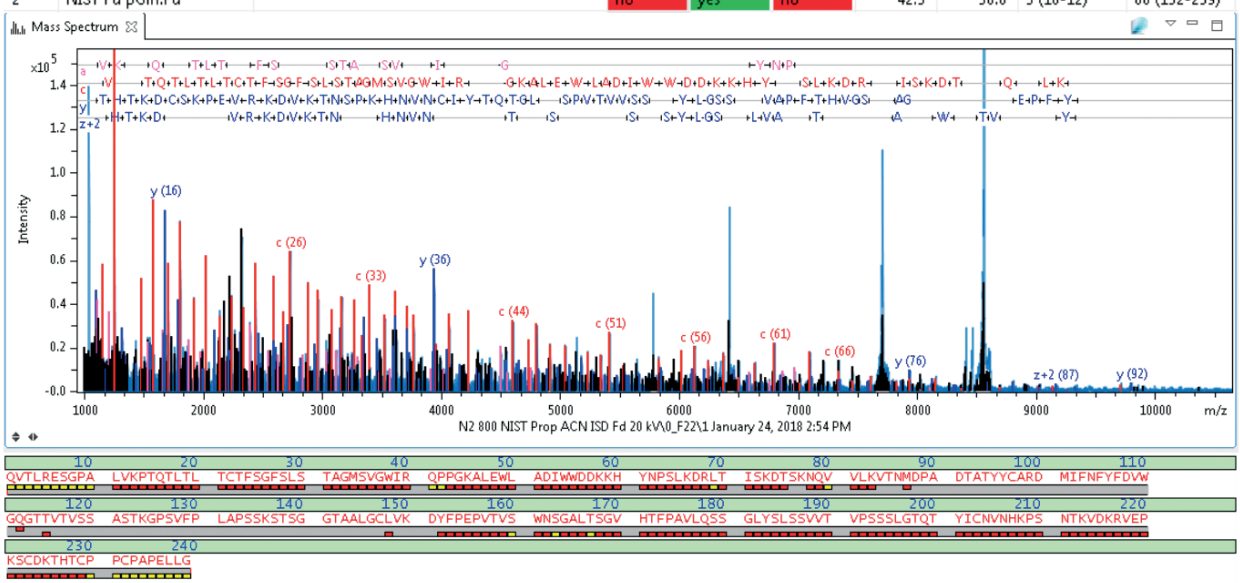

Figure 5: MALDI-TDS analysis of the Fd fraction. A sequence coverage of 61% and a sequence validation coverage of 72% was achieved, 2 out of 3 CDRs were covered by the analysis and confirmed. The top-panel verified the N-term of the Fd being pyro-glutaminated – the non-modified N-term sequence was not confirmed

Figure 6: MALDI-TDS analysis of the Fc/2 fraction confirming complete Lysine clipping at the C-terminus and G1F at N61 as an abundant glycan

TDS analysis of Fc/2 involved testing of 3 different variations: G0F and G1F as main glycans attached to N61 and Lys-clipping at the C-terminus. The C-terminal 86 residues were only confirmed assuming C-terminal lysine loss (Figure 6). In addition, the sequence modified with the G1F glycan at N61 was detected with an overall of 75 % SVP for this subunit and reading through the glycosylation site in contrast to the aglycon sequence and the G0F form in which sequences downstream the glycosylation site could not be observed. Thus, the data confirm the glycosylation site as well provide evidence for a higher abundant glycoform.

Conclusion

- For the LC-MALDI-TDS analysis on the NISTmAb we used the new enhanced workflow that was simplified dramatically comprising the nanoElute together with the new spotOn fraction collector, rapifleX MALDI-TOF/TOF mass spectrometer and a dedicated workflow in the BioPharma Compass 3.0 software.
- It was applied to confirm the terminal sequences up to 70-90 residues routinely, which was used for confirming large parts of the sequence in a single spectrum (~70-85%). For the LC, all three CDRs were confirmed, N-terminal pyro-glutamination of the Fd was detected and on the Fc/2 the glycosylation site and the C-terminal lysine clipping were determined. Similar results highlighting the unique capabilities of LC-MALDI-TDS for top-down protein sequence confirmation – also including analysis of further commercially available mAbs – were obtained in a recent ring study organized by the Consortium for Top-Down Proteomics [2] which currently is prepared for publication.
- The MALDI-TDS method has also been shown to be quite efficient in determining protein clipping variants and providing data qualities high enough for *de novo* sequencing of entirely unknown sequences [3].

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