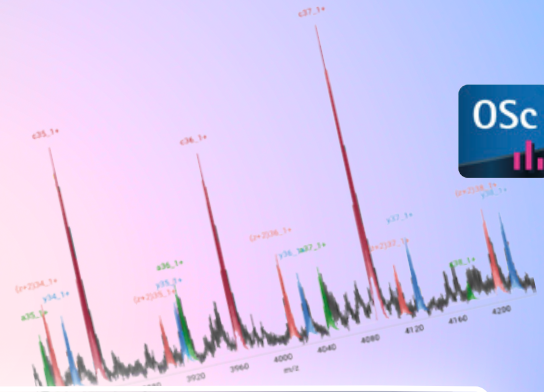


LSASV GDRVT ITCSA SSRVQ
SKLAS GVPSR FSGSG SGTEF
SGYPF TFGGG TKVEI KRTVA
VVCLL NNEYP REAKV QWKVD
TYSLS STLT L SKADY F



A Novel Top-Down Protein Sequencing Workflow Employing neofleX Benchtop MALDI-TOF/TOF and OmniScape Software

MALDI Top-Down Sequencing (MALDI-TDS) delivers N- and C-terminal sequence readout for intact proteins or protein subunits within minutes. MALDI-TDS enables unparalleled sequencing depth (typically up to 100 amino acid residues from both termini) including PTM assessment and verification of terminal status through easy-to-analyze singly charged MALDI-In-Source Decay (MALDI-ISD) fragmentation data and T³-Sequencing, i.e. pseudo MS³ of selected N- and C-terminal ISD fragments.

Abstract

The neofleX™ benchtop MALDI-TOF/TOF is an ideal MALDI-TDS platform producing high-quality top-down data. Using the OmniScape™ software, only few mouse clicks are required to turn raw MS data into meaningful sequencing results. Altogether, this provides for a novel MALDI-TDS workflow yielding detailed information regarding protein sequence, terminal status and modifications with shortest time to result. For the well characterized NIST reference monoclonal antibody (NISTmAb), a sequence coverage of 87% was achieved by MALDI-TDS analysis of its reduced and IdeS digested subunits, and five of the antibody's six CDRs were confirmed.

Keywords:
neofleX, OmniScape, MALDI-TOF/TOF, MALDI-TDS, MALDI-ISD, T³-Sequencing, Top-Down, characterization, protein, antibody, biopharma

Hence, the neofleX MALDI-TDS workflow provides a simple and fast tool for confident sequence verification enabling zero-delay decision-making in protein development and production in biopharma, bioengineering and other branches of industry.

Introduction

MALDI Top-Down Sequencing (MALDI-TDS) offers several unique advantages when compared to other protein characterization methods. In contrast to bottom-up methods, which commonly rely on the use of multiple proteases and extensive LC separation of the resulting peptide mixtures yielding large amounts of MS/MS data, MALDI-TDS is a much faster approach that analyzes intact proteins or protein subunits producing small datasets that are easy to analyze. In comparison to Top-Down ESI-MS/MS methods, where fragment ions of multiple charge states add to spectral complexity, MALDI-TDS stands out in that it analyzes singly charged fragment ions resulting from MALDI In-Source Decay (MALDI-ISD) [1,2]. Thus, MALDI-TDS data allow for simple and straightforward sequence readout of a target protein to be verified regarding amino acid sequence, terminal status and modifications.

Built on the unique merits outlined above, MALDI-TDS has a strong track record of successful application to the characterization of therapeutic proteins, for example monoclonal antibodies, nanobodies, bacterial glycoconjugates and PEGylated peptide drugs, but also other proteins, such as antigens and small membrane proteins [3-9].

neofleX benchtop MALDI-TOF/TOF provides an ideal MALDI-TDS platform combining efficient lab space usage with outstanding performance previously unseen on a benchtop instrument. Integrated smartbeam 3D laser technology enables fast analysis speed and optimum MALDI-ISD fragmentation efficiency. With a resolving power of up to $R=30,000$, neofleX is capable of acquiring isotopically resolved MALDI-ISD-MS spectra over a wide mass range resulting in sequence readout of 70-100 amino acid residues from both N- and C-terminus. neofleX's outstanding MS/MS performance allows for high-quality T³-Sequencing, i.e. pseudoMS³ analysis of selected MALDI-ISD fragments, enabling near-terminal sequence confirmation that is not directly accessible by MALDI-ISD. [10]

While MALDI-TDS datasets are typically of small file size, the rich information contained in imposes the need for sophisticated data interpretation software. OmniScape software provides the tools required to get the most out of neofleX MALDI-TDS data and can turn raw MS data into confident sequence readout with only few mouse clicks.

In this application note, the neofleX MALDI-TDS workflow is showcased by applying it to sequence verification of the NIST reference monoclonal antibody (NISTmAb). For the reduced mAb's heavy and light chains (HC, LC), MS/MS sequence coverage of 40 and 92%, respectively, was achieved. Coverage of the heavy chain increased to 84% when analyzing it at the domain level. Five of the mAb's six CDRs were successfully verified by the MALDI-ISD data. T³-Sequencing of selected N- and C-terminal ISD fragments provided additional proof for heavy chain N-terminal pyroGlu modification and C-terminal lysine loss. Overall, the MALDI-TDS dataset consisted of only 10 spectra (4 MALDI-ISD spectra of LC, HC, Fd, Fc/2; 6 T³-MS/MS spectra) resulting in a total data volume of less than 100 MB.

Methods

NISTmAb (NIST8671, Merck, Darmstadt, Germany) heavy and light chain (HC, LC) were released under denaturing conditions (GuaHCl) using DTT (both chemicals from Merck) as a reducing agent. NISTmAb subunits were generated by cleavage with IdeS (Genovis AB, Kävlinge, Sweden) and deglycosylation with IgGZERO (Genovis) followed by reduction with DTT under denaturing conditions as outlined above.

Reduced subunits were separated by C4 reversed phase liquid chromatography (Elute, Bruker Scientific, Billerica, MA, USA, equipped with Azura UVD 2.1S, Knauer, Berlin, Germany) applying a short 10 minutes acetonitrile gradient. Eluting NISTmAb chains or subunits were collected into Eppendorf tubes, dried down and reconstituted in TFA 0.1% in water.

Reconstituted protein samples were prepared for MALDI-TDS analysis on a Bruker MTP Anchorchip 384 BC MALDI target plate using 2,5-DHAP matrix (intact-mass analysis) and SDHB matrix (MALDI-MS; T³-Sequencing). MALDI matrices were all from Bruker.

MALDI analyses were performed on a Bruker neoflex benchtop MALDI-TOF/TOF instrument in positive ion mode. Intact-mass analysis was performed in linear operation mode. MALDI-MS data were acquired in reflector MS mode. T³-Sequencing spectra of selected N- and C-terminal ISD fragments were recorded in MS/MS mode. All data were acquired using default application methods. MALDI-MS and MALDI-MS/MS spectra were calibrated using bovine carbonic anhydrase II (Merck) as a calibrant.

Data were pre-processed (smoothing; baseline correction) in FlexAnalysis software and exported as ASCII files (*.txt) for further processing and interpretation in OmniScape.

MALDI-MS and T³-Sequencing spectra were analyzed in OmniScape software applying the workflows "Confirmation" and "Result Combination," respectively. In the "Confirmation" workflow, peak picking was performed by means of OmniScape's proprietary OmniWave™ algorithm, and MALDI-TDS spectra were matched against target protein sequences. The "Result Combination" workflow allowed for compiling MALDI-MS with multiple T³-Sequencing analyses providing a complete sequence coverage.

Results

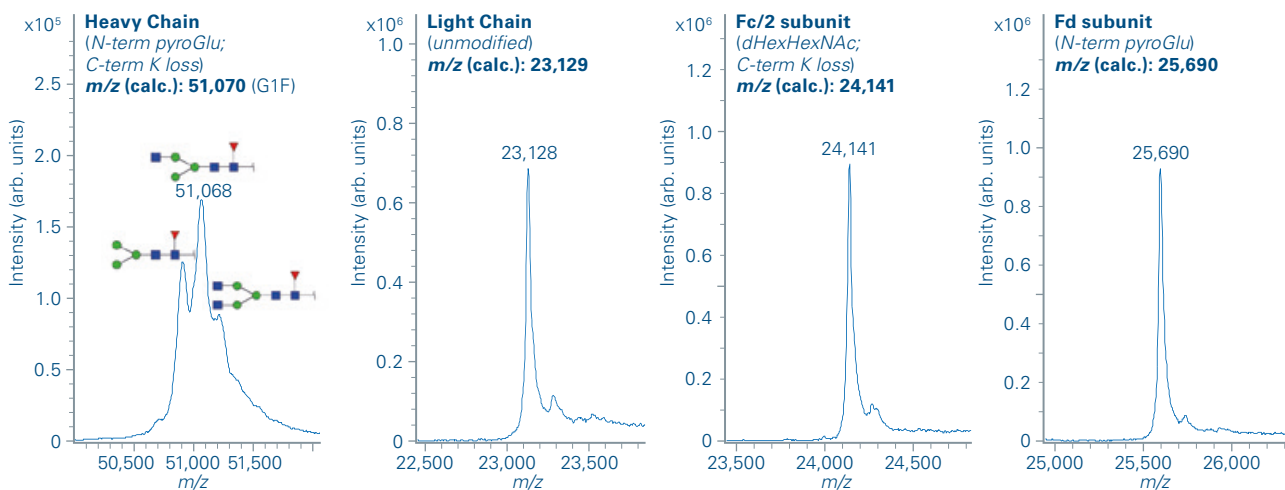


Figure 1

Intact-mass analysis of NISTmAb LC and HC as well as the deglycosylated Fc/2 and Fd

For the HC, data confirm three major N-glycoforms G0F-G2F, N-terminal pyroGlu and C-terminal lysine loss.

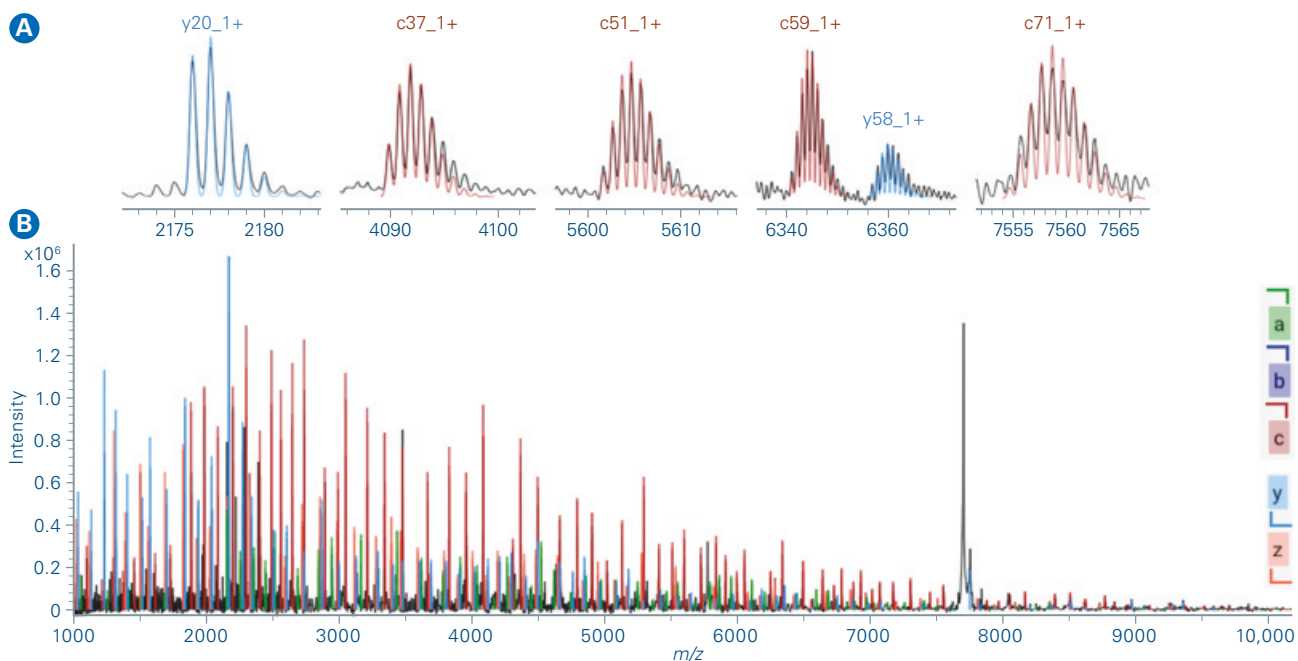


Figure 2
MALDI-MS/MS spectrum of NISTmAb light chain matched against LC sequence.
(A) Zoomed view on individual fragment ion signals. neoflex MALDI-MS/MS spectra feature isotopic resolution over a wide m/z range enabling high-confidence sequence readout. **(B)** Spectrum annotation in Omniscape. Annotation color codes for the individual fragment ion series.

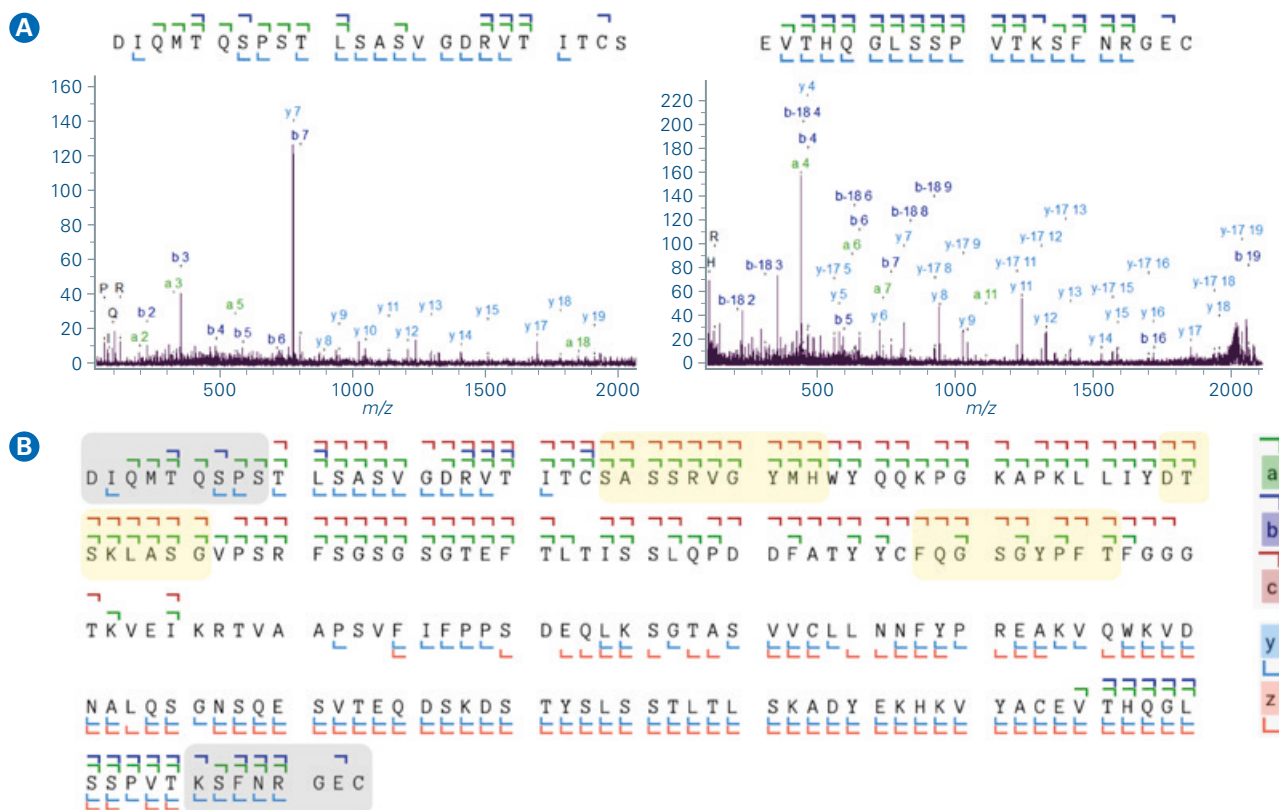


Figure 3
MALDI-TDS sequence verification of NISTmAb LC.
(A) T³ spectra acquired from MALDI-MS/MS fragments c24 (left) and y20 (right) confirm the absence of N- and C-terminal modifications. **(B)** Sequence map indicating a total sequence coverage of 92.5% obtained from MALDI-MS/MS (shown in Figure 2) and T³-Sequencing data. All three CDRs on the NISTmAb LC (labeled yellow) were covered by the MALDI-MS/MS data. Sequence information for the near-terminal LC regions (labeled grey) was retrieved from T³ data.

	MS/MS Seq. Coverage [%]	Terminal status as confirmed by MALDI-MS/T ³ -Sequencing	
		N-terminus	C-terminus
Light Chain	92,5	Unmodified	Unmodified
Heavy Chain	40,0	Gln->pyroGlu	Lysine loss
Fd subunit	83,6	Gln->pyroGlu	n.a.
Fc/2 subunit	84,2	n.a.	Lysine loss

Table 1

Summary of MALDI-TDS results obtained from reduced and IdeS cleaved NISTmAb. In total, the neoflex MALDI-TDS workflow verified 87% of the NISTmAb sequence. As the coverage values indicate, IdeS cleavage is a very efficient means to enhance sequence verification for the heavy chain (coverage more than doubled when compared to MALDI-TDS of intact heavy chain).

Conclusion

- neoflex benchtop MALDI-TOF/TOF and OmniScope software make a complete solution for simple and fast top-down sequence analysis of intact proteins or protein subunits.
- neoflex offers research-grade MALDI-TOF/TOF performance in true benchtop format and delivers high-quality MALDI-MS and T³-Sequencing data in minutes.
- OmniScope software provides the analysis tools required to get the most out of neoflex MALDI-TDS data turning raw spectra into confident sequence readout with just a few mouse clicks.
- For a well characterized mAb, the neoflex MALDI-TDS workflow verified 87% of the sequence including N- and C-terminal status, and covered 5 out of 6 CDRs. All this information was extracted from a dataset comprising 10 spectra (4 MALDI-MS spectra; 6 T³-MS/MS spectra) with a total data volume of less than 100 MB.
- The novel MALDI-TDS workflow described here provides for simple and fast verification of protein expression products enabling zero-delay decision-making in biopharma, bioengineering and other branches of industry.

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