Application Note # ET-17 / MT-99

Characterization of the N-glycosylation Pattern of Antibodies by ESI - and MALDI mass spectrometry

Abstract
Analysis of the N-glycosylation pattern on antibodies is described using complementary mass spectrometric strategies based on both, top-down ESI-UHR-TOF and bottom-up LC-MALDI-TOF/TOF. Fast LC-ESI-UHR-TOF analysis, performed on the Bruker maXis™, provides high-resolution, high-mass accuracy (confident low ppm) data for both, intact antibodies and released antibody heavy chains allowing a rapid assignment of the major N-glycosylation isoforms. Bruker’s new ultrafleXtreme™ platform facilitates in-depth characterization of the antibodies’ N-glycosylation patterns via LC-MALDI-TOF/TOF analysis of the N-glycopeptides generated from digested antibodies. The improved resolution provided by the instrument delivers an extremely detailed picture of the highly complex patterns of N-linked glycans present on antibodies from different origins. As a unique feature, MALDI-TOF/TOF data generated from N-glycopeptides, simultaneously provide information in the same spectrum including both the peptide sequence and the structure of the N-linked glycan moiety. Selective screening and subsequent analysis of spectra of N-glycopeptides from large LC-MALDI-MS/MS datasets is supported by dedicated features implemented in Bruker’s latest software tools.

Introduction
Antibodies represent one of the most important classes of glycoproteins playing a central role in the immune response of living organisms. Furthermore, there is a growing interest in recombinant antibodies as potential biotherapeutic agents. The detailed analysis of the N-glycosylation pattern present on antibodies is challenging due to the heterogeneous structure of this posttranslational modification. The glycan structure is highly dependent on the process by which the recombinant glycoprotein is generated, such as host organism and growth conditions. Changes to the glycosylation pattern can significantly alter biological function. To characterize the N-glycosylation pattern, various mass spectrometric techniques may be applied to analyze either the intact glycoprotein or the N-glycopeptides obtained from enzymatic digestion. We describe here the in-depth characterization of the N-glycosylation pattern in various antibody samples by utilizing two complementary MS based analysis techniques:
Fast top-down analysis based on LC-MS accurate mass measurement of the intact antibody and the released heavy chain, performed on the maXis; a next generation ESI-ultra high-resolution (UHR)-TOF instrument.

Comprehensive bottom-up characterization of the N-glycosylation patterns of various antibodies by nanoLC-MALDI-TOF/TOF analysis of N-glycopeptides obtained from enzymatically digested antibodies; employing the ultrafleXtreme MALDI-TOF/TOF instrument.

Experimental

The analyses described here were performed on three different samples: a human IgG1 expressed in Chinese hamster ovary cells, a commercially available bovine IgG, and a sheep IgG from prefractionated sheep plasma. The human IgG was reduced and alkylated to release the glycosylated heavy chain (HC). An aliquot of the sample was separated by SDS-PAGE. The gel-separated heavy chain was then subjected to in-gel digestion using trypsin as an enzyme. The bovine IgG was digested in-solution after reduction and alkylation. The sheep IgG was part of a pre-fractionated plasma sample that had been reduced, alkylated using MMTS, and, after tryptic digestion, had been labelled with the iTRAQ4plex label (Applied Biosystems).

The following experimental setup was used for the LC-ESI-UHR-TOF measurements on the intact glycoprotein:

<table>
<thead>
<tr>
<th>LC system</th>
<th>Agilent 1200 binary pump</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC column</td>
<td>Zorbax SBC8 Rapid Resolution Cartridge, 2.1 x 30 mm, 3.5 µm</td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.1% HCOOH in water</td>
</tr>
<tr>
<td>Solvent B</td>
<td>0.1% HCOOH in ACN</td>
</tr>
<tr>
<td>Gradient</td>
<td>0 min: 0%B, 3 min: 0 %B, 10 min: 100 %B, 13 min: 100 %B, 13.1 min: 0 %B</td>
</tr>
<tr>
<td>Column flow rate</td>
<td>300 µl/min</td>
</tr>
<tr>
<td>Column temperature</td>
<td>70°C</td>
</tr>
<tr>
<td>Mass spectrometer</td>
<td>Bruker maXis UHR-TOF MS, positive ion mode, internal calibration</td>
</tr>
</tbody>
</table>

Fig. 1: The upper panel shows the raw data as acquired on the Bruker maXis. The lower panel shows the spectrum after Maximum Entropy deconvolution using the mass range colored in gray on the raw spectrum. Considering average atomic weights from organic sources according to Zhang et al., the theoretical mass of the mass labeled glycosylation form is 149081.79Da, which deviates 2ppm from the measured mass.
The LC-MALDI-TOF/TOF analyses were performed under the following conditions:

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Specification</th>
</tr>
</thead>
<tbody>
<tr>
<td>LC system</td>
<td>Bruker EASY-nanoLC™</td>
</tr>
<tr>
<td>Trap column</td>
<td>Nanoseparations C18, 20x0.1mm</td>
</tr>
<tr>
<td>Analytical column</td>
<td>Dionex PepMapC18 150x0.075mm</td>
</tr>
<tr>
<td>Solvent A</td>
<td>0.05% TFA in water</td>
</tr>
<tr>
<td>Solvent B</td>
<td>0.05% TFA in 90% ACN</td>
</tr>
<tr>
<td>Gradient</td>
<td>2...45% B in 48min</td>
</tr>
<tr>
<td>Column flow rate</td>
<td>300 nl/min</td>
</tr>
<tr>
<td>Fraction collection</td>
<td>Bruker PROTEINEER fc II™, 192 fractions, 15s each</td>
</tr>
<tr>
<td>MALDI matrix</td>
<td>HCCA</td>
</tr>
<tr>
<td>Mass spectrometer</td>
<td>Bruker ultrafleXtreme TOF/TOF</td>
</tr>
</tbody>
</table>

Annotation of glycan structures and glycan specific fragments on MS and MS/MS spectra was carried out using Glycoworkbench software (www.eurocarbldb.org).

**Results**

Rapid assignment of major glycosylation isoforms

Figure 1 shows the data obtained from fast LC-MS analysis of the intact human IgG1. The ability of the Bruker maXis ESI-UHR-TOF instrument to generate high-resolution, highly accurate mass data of intact proteins allows a rapid assignment of major glycosylation isoforms of the analyzed human IgG1. Maximum Entropy deconvolution of the data yields a mass for the major glycosylation form, which fits very well to the expected average mass range based on a calculation of atomic weights from organic sources according to Zhang et al. (deviation between measured mass and expected mass based on average atomic masses from organic sources: 2ppm). Further glycosylation isoforms were assigned based on characteristic mass distances of 162Da indicating a rising number of galactose units. The released heavy chain of the intact human IgG was also analyzed by ESI-UHR-TOF (see Figure 2). The measured mass was found to be in excellent agreement with the expected mass, showing a 2ppm deviation from the calculated mass according to Zhang et al. In addition, the high-definition data provided by the maXis also allow unambiguous assignment of chemical artefacts which were generated during sample preparation. In the example shown here, spectral peaks that did not match the masses of expected protein sequence/glycosylation structures (denoted with * in Figure 2) could be assigned to non-desired by-products originating from the alkylation step, leading to an overalkylation, yielding additional peaks shifted in mass by +57Da.

In-depth characterization of the N-glycosylation pattern of various antibodies

Figure 3 shows as an example the LC-MALDI-MS chromatogram obtained from the analysis of bovine IgG. Applying multiple enzymes (trypsin, chymotrypsin, GluC, LysC), LC-MALDI analysis yielded, on average, a sequence coverage of 100% for the light chain and more than 98% for the heavy chain. As the glycan moiety does not contribute significantly to the specific retention of N-glycopeptides on a reversed phase column, N-glycopeptides appear at a relatively early retention time. This helps to separate the glycopeptides from non-glycosylated tryptic peptides,
particularly those with masses greater than 2500Da. Figure 4 shows the averaged LC-MALDI-MS spectrum of bovine IgG covering the N-glycopeptide retention time range (27...31min). The ultrafleXtreme MALDI-TOF/TOF typically achieves resolution values between 30,000 and 45,000 in the mass range of interest for the analysis of N-glycopeptides. This is beneficial in the avoidance of false annotations due to partially or non-resolved overlapping peaks, and contributes considerably to improving the significance of the results.

Figure 5 shows a MALDI-MS/MS spectrum of a bovine IgG N-glycopeptide at a parent m/z of 2780Da. The displayed spectrum clearly illustrates a unique feature of N-glycopeptide MS/MS spectra generated on a TOF/TOF system: Detailed structural information is provided on both the peptide as well as the glycan moiety in a single MS/MS spectrum. Most importantly, the mass of the peptide moiety can be read out directly from the spectrum. Using this mass as a “pseudo” parent mass, the peptide sequence can even be identified by means of a database search using MASCOT or alternative search engines. The sequence annotation of the spectrum was carried out using Bruker’s BioTools™ software. Furthermore, the fragment peak that corresponds to the peptide moiety is part of a pattern consisting of four peaks occurring in subsequent distances of 17/83/120Da. This peak pattern is highly specific to TOF/TOF spectra of N-glycopeptides, and originates from the fragmentation of the core HexNac unit that is attached to the peptide’s N-glycosylation site. This N-glycospecific fragment pattern can be used as a subject of a query to specifically extract from an extensive LC-MALDI-MS/MS dataset all those MS/MS spectra which potentially originate from N-glycopeptides. The structural annotation of the glycan fragments was performed using the “Glycoworkbench”, a software tool developed and made available to the public by the EurocarbDB project (http://www.eurocarbdb.org).

Based on the LC-MALDI-MS and – MS/MS data, the detected patterns of N-glycopeptides can now be assigned in detail. This is shown in Figure 6 for three different trypsin digested antibody samples, a bovine IgG, a sheep IgG, and a human IgG1 expressed in Chinese hamster ovary cells, respectively. The three samples described here, according to their individual origins, yield N-glycosylation patterns of very different complexity, including complex and high-mannose glycan structures. These patterns consist of at least two overlapping series of glycopeptides, one of them containing a missed trypsin cleavage site. When comparing the human IgG data generated by either top-down ESI measurement or bottom-up LC-MALDI analysis (see Figure 2 and Figure 6c, respectively), the relative abundance of the major glycosylation isoforms are in very close agreement with one another.

Conclusions

LC coupled to either ESI-UHR-TOF or MALDI-TOF/TOF represent complementary techniques offering unique capabilities for the characterization of the N-glycosylation pattern present in antibodies. The methods described here serve as powerful tools for rapid quality control and comprehensive characterization of glycoprotein drugs. LC-ESI-UHR-TOF performed on Bruker’s maXis instrument is a fast top-down method to provide exact mass data (mass accuracy in low ppm range) of antibodies in both, intact and reduced form. This data enables an instant assignment of major N-glycosylation isoforms, including the characterization of processing artefacts (e.g. non-desired protein overalkylation). The ultrafleXtreme TOF/TOF is a superior platform for the in-depth analysis of N-glycopeptides from digested antibodies. The extraordinarily high resolution provided by the instrument is of great benefit to tackle the complex heterogeneity of N-linked glycans. LC-MALDI-TOF/TOF provides information-rich, but easy-to-interprete MS and MSMS spectra of N-glycopeptides, yielding a detailed picture of the N-glycosylation pattern. MALDI-TOF/TOF data of N-glycopeptides simultaneously provides information on both the peptide sequence and the glycan structure in the same spectrum.

Novel features have been designed into Bruker’s MALDI software suite to support selective filtering of MS/MS spectra of N-glycopeptides out of large LC-MALDI-MSMS datasets, and to enable efficient subsequent analysis of this data to ultimately allow the annotation of highly heterogeneous N-glycosylation patterns.
High resolution N-glycopeptide analysis

Fig. 4: Averaged LC-MALDI-TOF spectrum covering the retention time range of N-glycopeptides from bovine IgG. Data was acquired on a Bruker ultrafleXtreme system achieving extremely high resolution over a wide mass range. The signals occurring in the spectrum reflect the structural heterogeneity of the N-glycosylation of the antibody.

Detailed structural information on the peptide and the glycan moiety

Fig. 5: MALDI-MS/MS spectrum of N-glycopeptide EEQFNSTYR from bovine IgG. From one and the same MS/MS spectrum, detailed information can be derived on the peptide mass, peptide sequence and the glycan structure, respectively.
References


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protein sequencing

Instrumentation & Software

maXis
ultrafleXtreme
EASY-nano LC
PROTEINEER fc II
BioTools

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