Direct Characterization of LMW Impurities using MS

Heiner Falkenberg, Daniel Waldera-Lupa, Sylvia Aretz-Meyer, Eva Ennemann, Anke Schnabel, Rebecca Clemens, Roland Moussa

ProtaGene GmbH, Dortmund, Germany

ProtaGene
Analytical Excellence

Introduction

The characterization of modern biopharmaceuticals is challenging, as the proteins show a large heterogeneity not only regarding modifications, but also in charge, conformation and microheterogeneity. Changes within this composition can have a large impact on efficacy and safety. In particular for the prove of biosimilarity the knowledge about quality attributes is crucial. Current methods are able to monitor single attributes of the complex composition (HPLC, CE, CGE, CZE). Combination of methods like SEC-MS, IEX-MS promise to monitor multiple critical quality attributes at one time and might give more information in less time and effort. Coupling of chromatographic separation techniques with high-resolution mass spectrometry offers the possibility to get information e.g. on the charge distribution and to directly characterize the peaks regarding lysine truncation. Another technique to characterize charge variants is CZE-MS using a ZipChip Device. This method is used to characterize stressed samples and to monitor the amount of oxidized species.

Low molecular weight (LMW) heterogeneity of biopharmaceuticals is typically characterized by CGE. CGE has the ability to separate LMW species of an antibody like H2L, LC, 2HC but is unfortunately incapable of being coupled to MS.

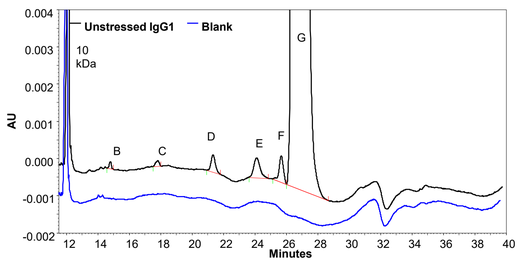

Figure 1: Classical CGE for separation of LMW species. The peaks are numbered according to their elution order. An assignment to a specific LMW species is not possible. Shown here is the separation of a marketed IgG1.

- CGE (or CE-SDS) is able to separate LMW species
- Assignment of signals is performed according to the elution order (molecular weight)
- Additional characterization of peaks is missing
- Coupling of CGE to MS is not possible
- Fractionation of CGE is difficult to perform and has low yields
- HILIC-MS generates a similar elution order like in the CGE
- Coupling to MS enables full characterization of single peaks

Methods

Here, we used hydrophilic interaction chromatography (HILIC) coupled to MS to achieve a separation similar to CGE for intact deglycosylated antibodies.

A marketed antibody was stressed and analyzed by HILIC-MS. Partially reduced stress sample generates all types of LMW. H2L, half-bodies, 2HC, HC and LC species were chromatographically separated. The UV-signal allows quantitative evaluation of LMW species. By coupling to MS, we were able to generate high resolving mass spectra allowing the unambiguous identification of LMW species. The LMW species were fractionated and visualized with 1D-PAGE as well as characterized by reinjection into CGE.

Vanquish Horizon | Q Exactive HF-X | PA800 Plus

HILIC-MS of deglycosylated IgG1

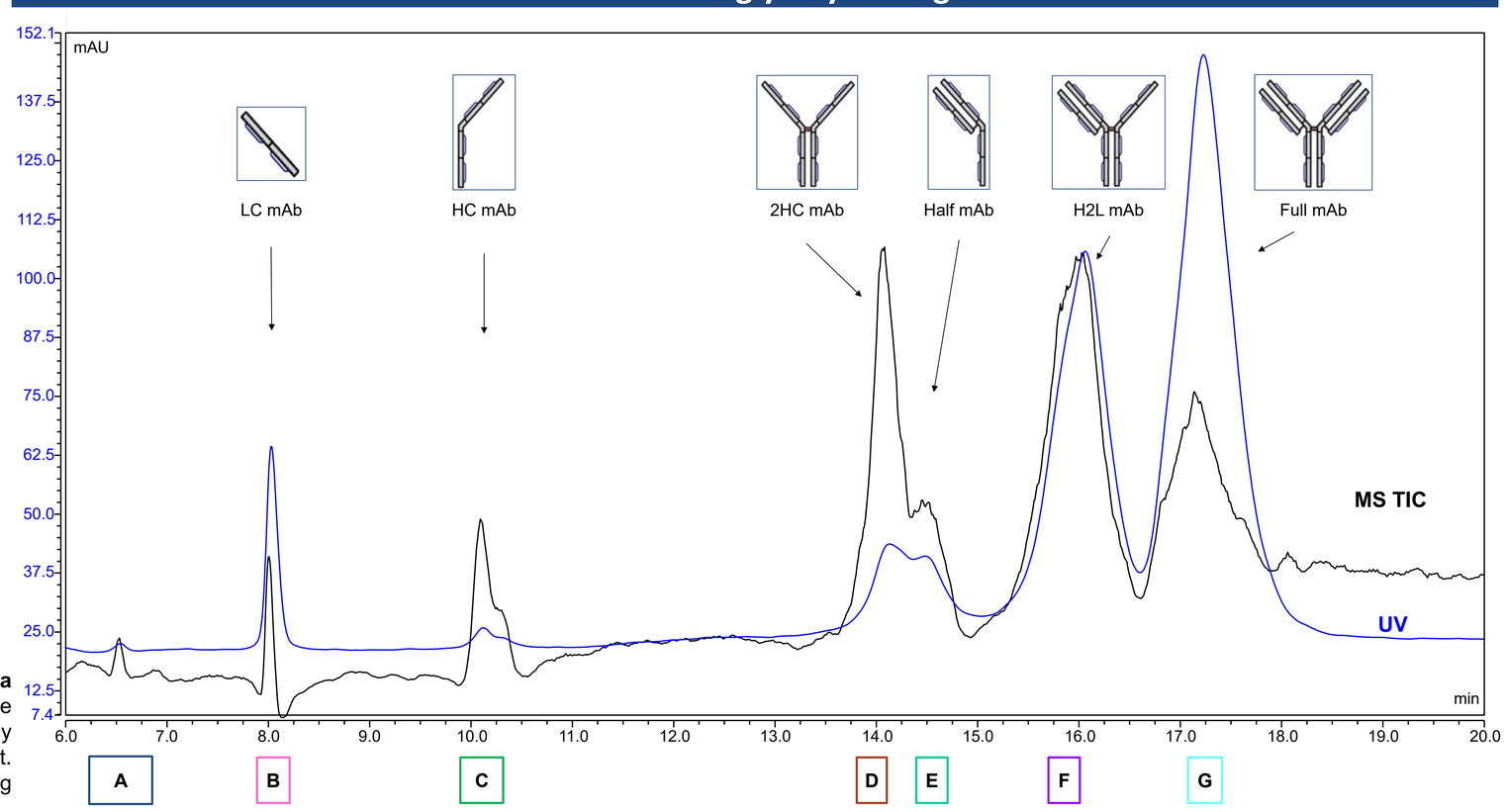

Figure 2: HILIC-MS separation of stressed, deglycosylated IgG1 showed a baseline separation of LMW species. Based on their molecular mass, the LMW species were assigned to antibody fragments. LC and HC were clearly separated. Interestingly, the halfbody eluted after the heavier 2HC-fragment. H2L and the full mAb could also be separated. The boxes below are showing the time frames for fractionation.

Fractionation of LMW Species after HILIC

The six detected LMW species and the main peak separated by HILIC were fractionated manually according to the timeframes given in Figure 2. The fractions were checked on a 1D-SDS-PAGE under non-reducing conditions for purification (Figure 3).

The fractions of a second purification where dried, solved in CGE compatible buffer and injected onto classical CGE (Figure 4). Here, the fractions were assigned to the LMW species in the unstressed sample.

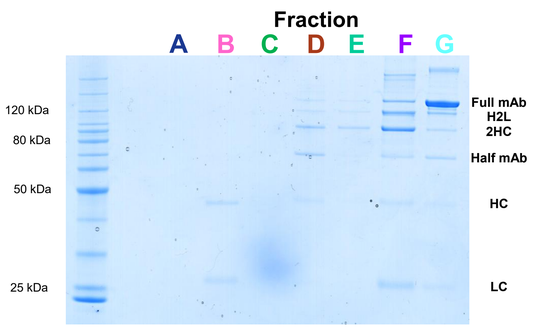

Figure 3: 1D-SDS-PAGE of HILIC-fractions under non-reducing conditions. The heavy chain was also detected in fraction B. The main species (full mAb, H2L2) was mainly found in fraction B, while the 2HC compound was found in fraction F.

CGE of HILIC-fractions

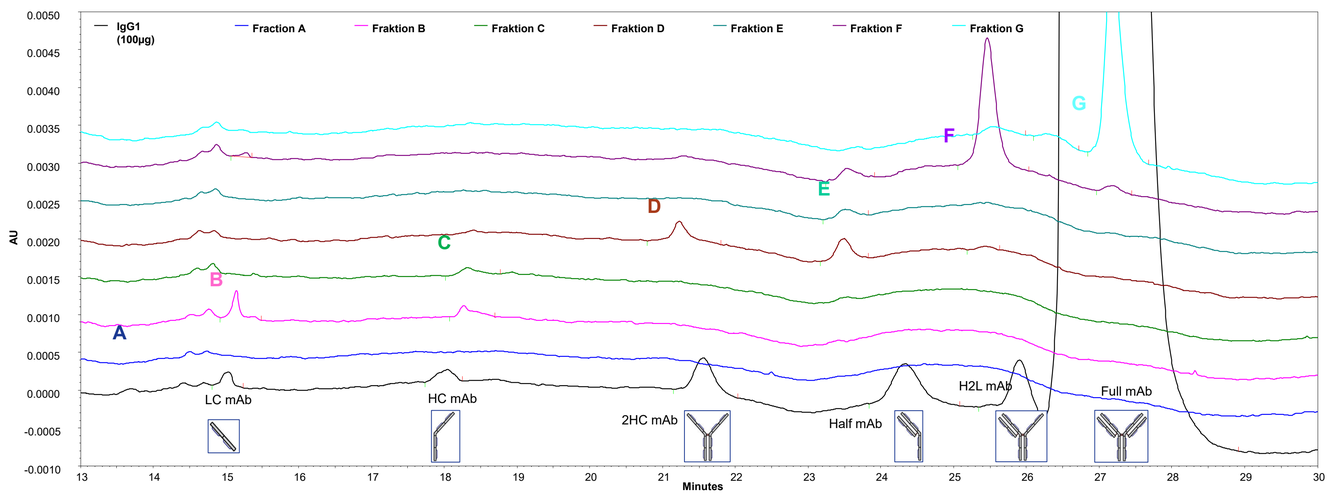

Figure 4: Electropherogram of HILIC-fractionated LMW-species. Each fraction could be unambiguously assigned to a single LMW-species. The CGE-profile is comparable to the unstressed, non-fractionated antibody (black). A slight shift in retention time was observed for all heavy-chain-fractions, because the unstressed sample was not deglycosylated before analysis. The LC fraction showed no shift.

Summary – HILIC-MS for the characterization of LMW impurities

- HILIC is able to separate LMW species.
- HILIC-MS is able to characterize all possible LMW species.
- HILIC-MS is able to compare the abundance of LMW species between different samples. This is necessary for example in Stability Testing Programs.
- Additionally, the peaks can be fractionated. This enables a re-injection into CE to confirm single signals in the CGE electropherogram.
- HILIC-MS supports CE as a classical release methods, e.g. for impurity characterization
- Further characterization using various methods is possible (PTM analysis, Cysteine Linkage Analysis, SEC)

Analysis of stressed (deamidated) antibody

Quantification of deamidation is a bottleneck in the analysis of intact protein analysis per LC-MS. Therefore, peptide-mapping MS after enzymatic digestion is one approach to quantify deamidation site-specifically.

Here, we used a CZE-MS approach to separate the deamidated species from the main species to characterize the deamidation profile. A high pH stressed antibody (IgG1) was analyzed using a ZipChip Devices coupled to a QExactive HF-X. The results were compared with site-specific data from peptide-mapping MS.

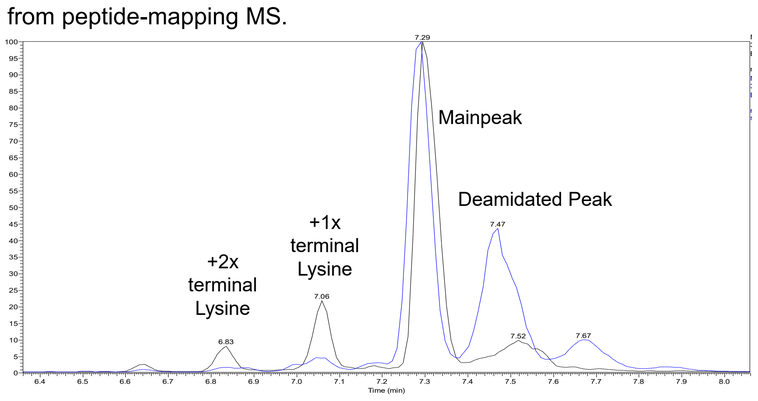

Figure 5: MS-signal (BPC) of unstressed IgG1 (black) and pH-stressed IgG1 (blue) after CZE-MS. The separation is based on the charge profile of the IgG1.

ZipChip Device coupled to a Q Exactive HF-X

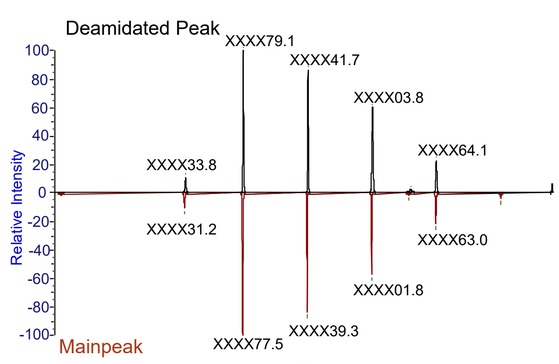

Figure 6: MS-spectra of the Mainpeak (lower) and the deamidated Peak (upper). The mass shift of 1-2 Dalton is a clear indication for deamidation. This shift is only visible in the intact mass analysis, because the deamidated peak is separated, otherwise the deconvolution algorithm would not be able to resolve the deamidation.
The distribution of the glycosylations are similar.

Table 1: Amount of modified IgG1 after high-pH stressing. The amount of deamidated proteins quantified using CZE-MS based on peak-area is similar to the results of a peptide-mapping MS approach.

	CZE-MS [based on peak area]		SeqPTM [summed over all sites]	
	unstressed	stressed	unstressed	stressed
Deamidation	6%	39%	8%	32%
0K	82%	93%	96%	97%
1K	14%	4%	4%	3%
2K	4%	3%	n/a	

- CZE-MS is able to separate Lysine-truncated forms and deamidated forms
- CZE-MS enables quantification of deamidation on the intact, glycosylated antibody with similar results as the site-specific PTM-mapping.