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Disulfide Bond and Glycosylation Site Occupancy Mapping of Trastuzumab Using a Novel Capillary MAbPac RP Column

Zoltan Szabo[1], Xuefei Sun[1], Brandon Robson[1], Mike Baynham[2] and Shanhua Lin[1]
[1]Thermo Fisher Scientific, Sunnyvale, USA, [2]Thermo Fisher Scientific, Runcorn, UK

ABSTRACT

Purpose: The novel capillary Thermo Scientific™ MAbPac™ RP column (4 µm, 0.15 mm x 150 mm) developed originally for intact protein analyses has proven to be an excellent tool for peptide mapping of tryptic digest of therapeutic antibodies. This research aims at demonstrating the utility of this column for mapping some Post Translational Modifications (PTMs) at peptide level.

Methods: Intact mAbs were digested using a Thermo Scientific™ SMART Digest™ Trypsin Kit. In disulfide bond mapping experiments, unreduced and reduced digests were compared. Using heavy ($H_2{}^{18}O$) water enabled to identify glycosylation site occupancy and chemical deamidation. Peptides were separated and analyzed in data dependent acquisition using a Thermo Scientific™ UltiMate™ 3000 RSLCnano System coupled to a Thermo Scientific™ Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer controlled by Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) and Thermo Scientific™ Xcalibur™ software.

Results: Highly reproducible peptide mapping method resulting in almost 100 % sequence coverage using only 100 ng digest enabled to identify all the disulfide bonds and led to confident, reproducible characterization of site occupancy of mAbs.

INTRODUCTION

Peptide mapping is an important step during biotherapeutic characterization. From drug discovery to bioproduction, peptide mapping is one of the key analytical tools. A general peptide mapping workflow consists of four essential steps beginning with the digestion of proteins with sequence specific endoproteinases such as trypsin, Lys-C or Glu-C, etc., followed by peptide separation by HPLC, mass spectrometric data acquisition of the separated peptides and finally data analyses using a peptide mapping software.

Monitoring of PTMs in therapeutic proteins to ensure batch to batch reproducibility, efficacy and safety of the drug materials is necessary and required by different regulatory agencies. Some of the PTMs such as disulfide bond formation or glycosylation have crucial roles in the folding of proteins into their final three dimensional, biologically active conformation. Deamidation, a conversion of asparagine or glutamine into the corresponding aspartic acid or glutamic acid is conjectured to be one of the limitations of the useful lifetime of therapeutics. Aside from the importance of determining glycoforms and glycosylation analyses, measuring glycosylation site occupancy of drug materials and following up therefore on consistency across different batches is also an important quality attribute required by biopharmaceutical industry.

MATERIALS AND METHODS

Sample Preparation

Trastuzumab (mAb) was used in this work. mAbs was digested applying Thermo Scientific™ SMART Digest™ Trypsin Kit, the digestion in general was followed by reduction with TCEP (Thermo Scientific™, (Bond-Breaker™ TCEP solution, Neutral pH) and alkylation with iodoacetamide (Acros Organics, Fisher Scientific™) except for unreduced digest at disulfide mapping. Labeling reaction of reduced and alkylated peptides with heavy ($H_2{}^{18}O$) water (Sigma-Aldrich) to discriminate masses was carried out according to [1]. PNGase F was a Rapid™ PNGase F kit (New England Biolabs Inc, Ipswitch, MA).

Chromatography and mass spectrometry

In capillary chromatographic experiments, mobile phase “A” was water containing 0.1 % formic acid and mobile phase “B” was 80/20 % acetonitrile/water (v/v) containing 0.1 % formic acid (by volume). The flow rate applied was 2 µL/min. The gradient started at 5 % “B” and increased to 50 % “B” over 30 min. Analyses of peptides were performed at 45 ºC.

Tryptic peptides were analyzed in data dependent acquisition mode (Full MS-ddTop5) in positive mode. The spray voltage was 1.5 kV and the capillary temperature was set at 320 ºC. MS^1 spectra were collected in the scan range of m/z 350-1200 at 70 000 resolution, the AGC target in MS^1 data acquisition was set to $3x10^6$ at 60 ms maximum injection time. Data dependent MS^2 experiments for top 5 ions were carried out using 28 kV normalized collision energy at 17,500 resolution and 80 ms injection time. The minimum AGC target and threshold were set at $2x10^3$ and $2.5x10^4$. the quad isolation window was 1.9 T. The number of transients were 1 in both MS^1 and MS^2.

Data Analysis

MS data were processed using Thermo Scientific™ BioPharma Finder™ 3.2 Software.

RESULTS

Figure 1 exhibits three separations of 100 ng of tryptic digest of Trastuzumab using different columns and injecting samples prepared at different times. Apart from the identical profiles and very similar retention times, sequence coverage obtained for the light chain was identical in the three experiments, resulting in 98.1 %. Slightly lower but still acceptable values were achieved for the heavy chain. The XICs of the three peptides containing 7, 19 and 26 amino acids are depicted by Figure 2. Peak width measured at the half height slightly increased from 0.12 min to 0.15 min as the gradient progressed in 15 min. This indicates that no significant peak broadening was observed which makes this column a good tool for peptide mapping.

Figure 1. Highly Reproducible Analyses of 100 ng Tryptic Digest of Trastuzumab on MAbPac RP Capillary Column

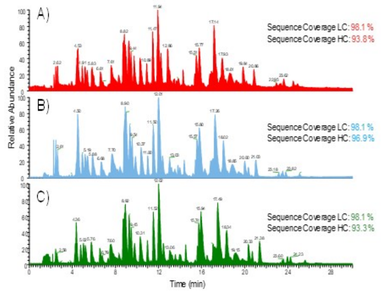

Figure 2. Reproducibility of Retention Time and Peak Width at Half Height for Tryptic Peptides at Different Length

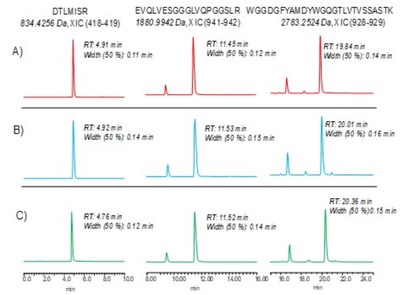

Figure 3. Disulfide Bond Mapping of Trastuzumab by Comparing Unreduced and Reduced Samples

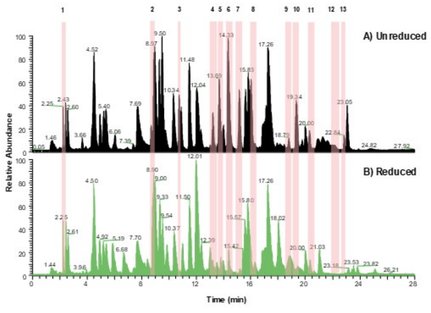

Figure 3/A is the TIC of 100 ng tryptic, unreduced digest of Trastuzumab. As a reference, 100 ng reduced, alkylated tryptic digest of the same mAb was compared to unreduced (Figure 3/B). Peaks appearing in Figure 3/A but not observable (or their intensity is significantly lower) in Figure 3/B are shown in the lanes numbered from 1 to 13. These peaks represent peptides with all the expected inter- and intrachain disulfide bonds (listed in Table 1). Except for peak 1 - which is the shortest disulfide containing peptide and includes an interchain disulfide bond between heavy and light chains-all the other disulfide containing peptides are represented by well separated peaks- which clearly highlights the outstanding resolving power of the column.

Table 1. Identified Disulfide Bond Containing Peptides in Unreduced Trastuzumab Digest

Number	Peptide Sequence	Theoretical m/z	Experimental m/z	Error, ppm	Cysteine linkage	Type of disulfide bond
1	SFNRGEC/SCDK	1260.4860	1260.4841	-1.45	1:C214/2:C223	Light-Heavy, 1ss
2	TPEVTCVVVDVSHEDPEVK/CKVSNK	2756.3359	2756.3381	0.80	2:C264/2:C324	Heavy-Heavy, 1ss
3	LSCAASGFNIK/AEDTAVYYCSR	2384.0779	2384.0789	0.41	2:C22/2:C96	Heavy-Heavy, 1ss
4	NQVSLTCLVK/SRWQQGNVFSCSVMHEALHNHYTQK	4087.9570	4087.9624	1.31	2:C370/2:C428	Heavy-Heavy, 1ss
5	NQVSLTCLVK/WQQGNVFSCSVMHEALHNHYTQK	3844.8230	3844.8301	1.84	2:C370/2:C428	Heavy-Heavy, 1ss
6	SGTASVVCLLNNFYPR/HKVYACEVTHQGLSSPVTK	3820.9031	3820.9058	0.70	1:C134/1:C194	Light-Light, 1ss
7	LSCAASGFNIKDTYIHWVR/AEDTAVYYCSR	3454.6079	3454.6106	0.78	2:C22/2:C96	Heavy-Heavy, 1ss
8	SGTASVVCLLNNFYPR/VYACEVTHQGLSSPVTK	3555.7490	3555.7483	-0.51	1:C134/1:C194	Light-Light, 1ss
9	VTITCR/SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	5444.6571	5444.6523	-0.88	1:C23/1:C88	Light-Light, 1ss
10	VTITCR/SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	4819.2422	4819.2524	2.13	1:C23/1:C88	Light-Light, 1ss
11	DIQMTQSPSSLSASVGDRVTITCR/SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK	6679.1343	6679.1265	-1.18	1:C23/1:C88	Light-Light, 1ss
12	THTCPPCPAPELLGGPSVFLFPPKPK/THTCPPCPAPELLGGPSVFLFPPKPK	5454.7832	5454.7925	0.72	2:^C229;^C232/2:^C229;^C232	Heavy-Heavy, 2ss
13	STSGGTAALGCLVK/DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYICNVNHKPSNTKVDK	8259.1335	8259.1621	3.47	2:C147/2:C203	Heavy-Heavy, 1ss

Figure 4. Labeling with $H_2{}^{18}O$ Discriminates Masses and Helps Identify Glycosylation and Deamidation Sites

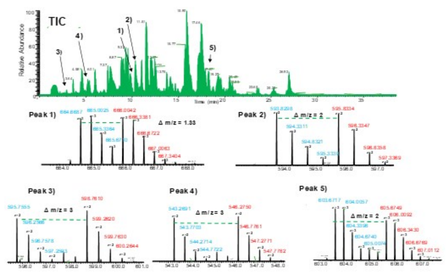

Figure 5. Calculation of Site Occupancy of *N300* in Trastuzumab

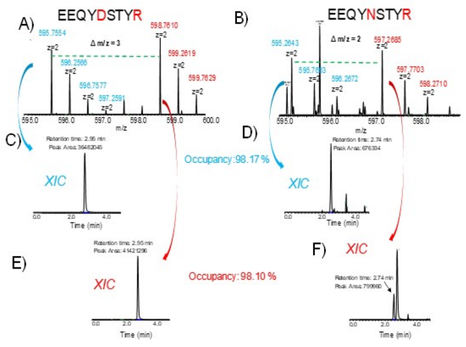

Figure 4 displays a TIC wherein 5 peaks are assigned, their spectra shown below. peak 1 is detected as a triply charged doublet, with the difference of m/z 1.33 between light and heavy isotopologues. This means only 4 Da difference between heavy and light parent ions of the peptide. However, simulated digestion of Trastuzumab *in silico* reveals that this peptide is expected to contain asparagine (N), contrary to the detected aspartic acid (D). The conversion of asparagine into aspartic acid occurred before the experimental procedure was carried out. Hence, this deamidation is probably attributed to process development of the drug. In contrast to this, peak 4 and 5 both exhibit 6 Da difference between the light and heavy parent ions in the corresponding doublets, indicating that the conversion of asparagine into aspartic acid took place during the experimental procedure, resulting in the incorporation of a heavy oxygen. Peak 3 is the peptide containing the glycosylation site (N300). As such, while deglycosylation with PNGase F, this asparagine was converted into aspartic acid (6 Da difference between the light and heavy labeled peptides). Calculation of site occupancy shown by Figure 5 is performed from the ratio of peak areas of both the light (C and D) and heavy (E and F) EEQYDSTYR (the peptide which was originally glycosylated) and EEQYNSTYR (the peptide which was not originally glycosylated in the drug material).

CONCLUSIONS

- Using MAbPac RP capillary column results Highly reproducible, high resolution separations of tryptic peptides of therapeutic antibodies enables to map PTMs such as disulfide bonds, deamidations and calculate glycosylation site occupancy

REFERENCES

1. Liu, Z.; Cao, J., He, Y.; Qiao, L.; Xu, C.; Lu, H.; Yang, P. Tandem ^{18}O Stable Isotope Labeling for Quantification of N-Glycome. *J. Proteome Res.* **2010**, 9, 227-236.

ACKNOWLEDGEMENTS

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TRADEMARKS/LICENSING

PO65768-EN 0422S

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