Peptide Mapping of a Monoclonal Antibody using a Microfluidic-based HPLC-Chip coupled to an Agilent Accurate-Mass Q-TOF LC/MS

Application Note

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Abstract

The sequence characterization of a monoclonal antibody (mAb) at low nanogram levels using an Agilent HPLC-Chip system coupled to an Agilent Accurate-Mass Quadrupole Time-of-Flight (Q-TOF) LC/MS instrument is described. The exact mass measurement provided by this high performance Q-TOF LC/MS ensures confidence when determining analytes such as peptides. The superior sensitivity and mass accuracy of the HPLC-Chip LC/MS platform, combined with the powerful data processing capabilities of Agilent MassHunter and BioConfirm software, enabled complete sequence coverage of heavy and light chains and enabled mass confirmation of all identified peptides within a 4 ppm mass accuracy. The robust, reliable, and easy to use Agilent HPLC-Chip/MS System, which has significant benefits for peptide analyses, is ideally suited for the routine analysis of small quantity biopharmaceuticals.

Introduction

The sequence confirmation of therapeutic monoclonal antibodies is of prime importance before product release. Peptide mapping using LC/MS is an established analytical tool for the confirmation of amino acid sequences of monoclonal antibodies [1]. In this application note, a purified mAb was subjected to proteolytic digestion followed by peptide separation and mass determination on a HPLC-Chip coupled to an Accurate-Mass Q-TOF LC/MS. The data obtained from LC/MS were analyzed using the powerful features of the MassHunter Qualitative Analysis software package. The MS results of the trypsin digest yielded 95% sequence coverage of heavy chains and 85% of light chains, and in combination with different proteases, 100% coverage of both chains. The matched peptides were searched at a mass accuracy of 5 ppm. Furthermore, the MS/MS results obtained for peptides confirm the peptide sequence of mAb. This method of using HPLC-Chip technology in combination with True High-Definition TOF technology for antibody characterization is a valuable method in the biopharmaceutical industry for QC studies. This application note continues our studies toward complete characterization of monoclonal antibodies using advanced Agilent platforms ideal for the biopharma market [2].

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Materials

Immunoglobulin G (IgG) was obtained from ProMab Biotechnologies, Inc. DL-Dithiothreitol (DTT), iodoacetamide, Tris (hydroxymethyl)-aminomethane (Tris Base), sequence grade Glu-C, and α-chymotrypsin were purchased from Sigma Aldrich. High quality sequence grade trypsin was obtained from the Stratagene division of Agilent Technologies.

Sample pretreatment for different protease reactions:

Reduction and alkylation of an antibody under denaturation conditions

Before the digestion of the mAb with various proteases, the disulfides were reduced and alkylated under denaturation conditions. This pretreatment ensured that the monoclonal antibody was completely denatured and soluble allowing the protease to access its substrate efficiently [3, 4].

The mAb was lyophilized and reconstituted in 2 µL of 8 M urea in 0.25 M Tris buffer, pH 7.6, containing DTT and incubated for 30 min at 37°C. 2 µL of a solution containing iodoacetamide in 8 M urea in 0.25 M Tris buffer, pH 7.6, was added and the sample was incubated at ambient temperature in the dark for 15 min. The solution was diluted with 160 µL of 0.25 M Tris buffer, pH 7.6, before digestion with various proteases.

Protease digestion

To the pretreated mAb solution, trypsin and α-chymotrypsin were added at a ratio of 20:1 and Glu-C at a ratio of 50:1 (protein to protease w/w). The reaction was incubated overnight for trypsin digestion, incubated 8 h for α-chymotrypsin digestion, and incubated 18 h for Glu-C digestion at 37°C before mass spectrometry analysis. The enzymatic activity was quenched by adding 1 µL of 10% formic acid solution. The samples were either immediately analyzed by LC/MS/MS or stored at -80°C until further LC/MS/MS analysis.

Figure 1: Microfluidic-based Chip system coupled to Q-TOF mass spectrometer.

Instrumentation

The Agilent 1200 HPLC-Chip/MS Interface (PN: G4240A) was coupled with the Agilent 6520 Accurate-Mass Q-TOF LC/MS platform for LC/MS analyses (**Figure 1**).

LC Parameters:

HPLC-Chip: 5 µm, ZORBAX 300SB-C18 (300Å), 40 nL enrichment column and a 75 mm x 43 mm analytical column (PN: G4240-62001).

Flow rate: 3 µL/min from Agilent 1200 Series Capillary Pump (PN: G1382A) to the enrichment column and 600 nL/min from Agilent 1200 Series nanoflow LC pump (PN: G2226A) to the analytical column.

Solvents: 0.1% formic acid in water (A); 90% acetonitrile in water with 0.1% formic acid (B). Flush volume was set at 4 µL.

Sample Loading: With Agilent 1200 Series Capillary Pump at 3% B.

Amount of sample injected onto the chip: 50 ng of the protein digest.

Sample analysis: Gradient with Agilent 1200 Series nanoflow LC pump as shown below.

Time (min)	B (%)
Initial	3
30	50
32	95
34	95
34.10	3

Stop time: 36 min

MS Parameters:

Spectra were recorded in positive ion and in centroid mode.

Vcap: 1900 V and drying gas flow of 5 L/min at 325°C was used.

Fragmentor voltage: 175 V
Data were acquired at high resolution (3,200 *m/z*), 4 GHz. For MS only mode, range 300–3,200 m/z. For MS/MS, spectra were acquired in auto MS/MS mode with the following parameters: MS scan, *m/z* 300–3,200 at 1 spectra/sec and MS/MS scan, *m/z* 50–3,000 at 3 spectra/sec. Precursor selection criteria: maximum of 3 precursors above TH 1,000, active exclusion after 2 spectra for 0.5 min, preferred charges 2, 3, >3, unk (unknown). Collision Energy (CE): 3.7 V/100 Da, 2.5 V Offset. An internal mass calibration sample was infused continuously during the LC/MS runs. This internal reference mass system ensured accurate, precise, automated mass accuracy measurements during the LC/MS runs.

Data analysis: Data was processed using Agilent MassHunter Qualitative Analysis software, Agilent MassHunter BioConfirm and Agilent Spectrum Mill MS Proteomics Workbench software packages.

Molecular feature extraction: The raw data (chromatograms) were processed using the Molecular Feature Extractor (MFE) program within Agilent MassHunter Qualitative Analysis software.

Define and match sequence: Both the light and heavy chain sequences were digested using trypsin with 2 missed cleavages to generate a theoretical peptide digest list. The compounds extracted using MFE were matched against this list.

Spectrum Mill MS Proteomics Workbench: Spectrum Mill MS Proteomics Workbench was used to create a user defined database that contained the sequence information of the antibody under study. The MS/MS data was searched against this artificial database to assign the peptide sequence.

Results and discussion

Peptide mapping is routinely used for the assessment of antibody quality. **Figure 2A** shows the base peak chromatogram (BPC) of trypsin digested mAb obtained using the HPLC–Chip/MS system for nanospray LC/MS. Inspection of the chromatogram reveals only peptide peaks with no undigested protein product. The extracted ion chromatograms (EICs) of some of the peptides for both light and heavy chains are shown in **Figure 2B**. These results show the high performance of HPLC-Chip separation, which is important in peptide mapping.

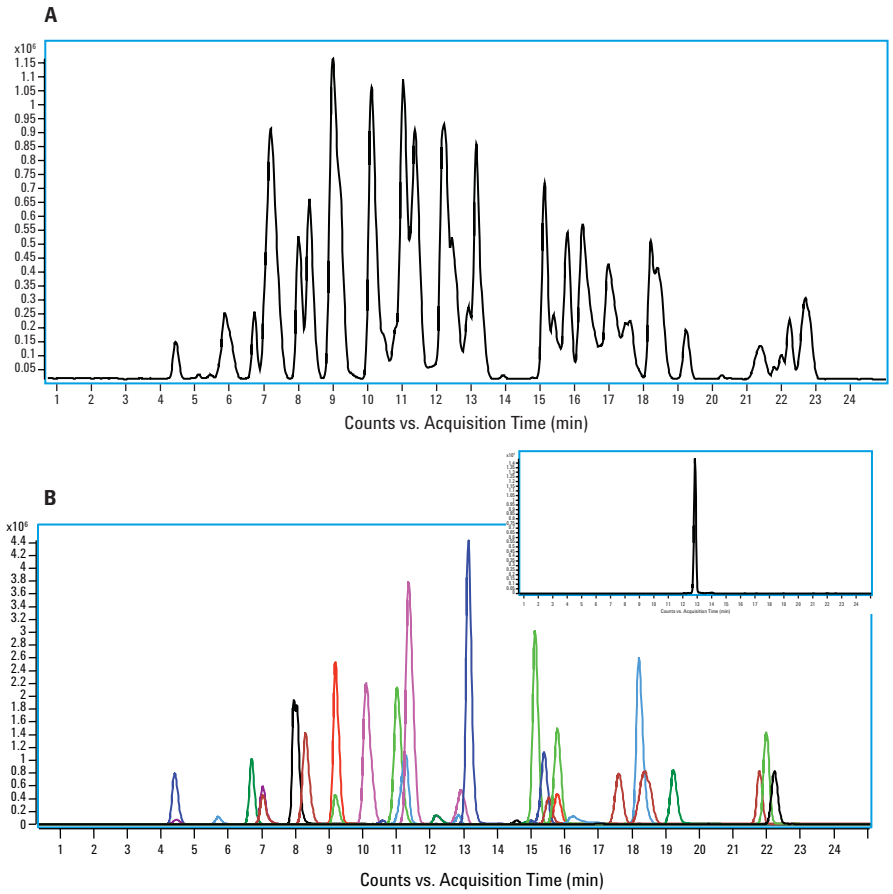


Figure 2: A. Base peak chromatogram (BPC) of trypsin digested mAb on a C18 HPLC-Chip.
B. Extracted ion chromatogram (EIC) of some peptides from mAb light chains and heavy chains. The inset shows the expanded view of the peak with a narrow width of ~ 6 sec.

MFE yielded peptide masses for the LC/MS run. The peptide masses obtained for the light and heavy chains were then matched with the theoretical digest using a 5 ppm error. The theoretical digestion list of peptides for the trypsin digested mAb was generated using the BioConfirm sequence editor (define and match sequences). The list of matched peptides is shown in **Table 1**. All of the identified peptides are within a 4 ppm mass accuracy. The measured average mass deviation was found to be 1.35 ppm.

A. Light Chain

Sequence Name	Label	Mass (Da)	Target Sequence Mass (Da)	Match Difference (ppm)
light chain	A(1-19)	1996.088	1996.0841	1.98
light chain	A(26-47)	2438.1986	2438.1979	0.31
light chain	A(48-56)	992.5656	992.5655	0.15
light chain	A(64-93)	3388.5238	3388.5194	1.31
light chain	A(94-107)	1538.7191	1538.7154	2.43
light chain	A(112-130)	2101.1245	2101.1208	1.76
light chain	A(113-130)	1945.0199	1945.0197	0.09
light chain	A(131-146)	1796.8922	1796.888	2.38
light chain	A(150-173)	2676.2669	2676.2627	1.55
light chain	A(150-187)	4160.01	4160.0033	1.61
light chain	A(154-173)	2134.9657	2134.9615	1.99
light chain	A(193-211)	2140.0778	2140.0735	1.99
light chain	A(195-211)	1874.9254	1874.9197	3.06

B. Heavy Chain

Sequence Name	Label	Mass (Da)	Target Sequence Mass (Da)	Match Difference (ppm)
heavy chain	A(1-19)	1880.0511	1880.048	1.65
heavy chain	A(20-38)	2126.9587	2126.9554	1.56
heavy chain	A(39-67)	2927.4492	2927.4414	2.68
heavy chain	A(39-65)	2714.3216	2714.3188	1.05
heavy chain	A(44-65)	2233.0584	2233.0539	2
heavy chain	A(44-67)	2446.1802	2446.1765	1.51
heavy chain	A(66-72)	835.4663	835.4664	-0.11
heavy chain	A(68-72)	622.3431	622.3439	-1.28
heavy chain	A(73-87)	1768.8812	1768.8778	1.93
heavy chain	A(77-87)	1324.6816	1324.6809	0.51
heavy chain	A(88-98)	1317.5937	1317.5911	2
heavy chain	A(99-105)	714.4018	714.4024	-0.94
heavy chain	A(106-129)	2531.2534	2531.2479	2.2
heavy chain	A(107-129)	2375.1509	2375.1468	1.74
heavy chain	A(130-141)	1185.6426	1185.6394	2.72
heavy chain	A(142-155)	1320.6705	1320.6708	-0.21
heavy chain	A(156-218)	6712.309	6712.3072	0.27
heavy chain	A(156-221)	7054.4995	7054.4975	0.28
heavy chain	A(156-222)	7182.5962	7182.5925	0.52
heavy chain	A(227-256)	3333.643	3333.6349	2.45
heavy chain	A(231-256)	2843.4544	2843.4503	1.45
heavy chain	A(257-263)	834.4274	834.4269	0.55
heavy chain	A(264-282)	2138.0249	2138.0202	2.22
heavy chain	A(283-296)	1676.7985	1676.7947	2.25
heavy chain	A(297-309)	3115.3418	3115.3315	2.13
heavy chain	A(310-325)	1807.0038	1806.9992	2.51
heavy chain	A(310-328)	2227.2034	2227.2001	1.5
heavy chain	A(335-342)	837.496	837.496	0
heavy chain	A(347-368)	2509.3347	2509.3289	2.32
heavy chain	A(349-368)	2310.1993	2310.1968	1.08
heavy chain	A(353-363)	1285.6677	1285.6667	0.79
heavy chain	A(353-368)	1871.9648	1871.9629	1.03
heavy chain	A(369-378)	1160.6228	1160.6223	0.4
heavy chain	A(379-400)	2543.1289	2543.1241	1.9
heavy chain	A(401-417)	1872.9184	1872.9146	2.06
heavy chain	A(423-447)	3043.3964	3043.393	1.12
heavy chain	A(425-447)	2800.2679	2800.2598	2.89
heavy chain	A(448-454)	659.3488	659.349	-0.35

Table 1: Peptide mass list after digestion of mAb with trypsin.

The matched peptides cover 95% of the heavy chains and 85% of the light chains. The BioConfirm tool enabled interpretation of the data in an easy and convenient way. In order to increase sequence coverage of the mAb, two more proteases were employed, Glu-C and α-chymotrypsin (data not shown). The results obtained from these digestions cover the sequences that were not covered earlier by trypsin digestion. This strategy of using combinations of two or more proteases helps with complete sequence characterization of the protein under study. This study (only MS) could also be achieved by using the HPLC-Chip technology coupled with a TOF analyzer such as Agilent's 6220 Accurate-Mass TOF LC/MS. However, for this study MS/MS data was also acquired as an example of further characterization of the mAb tryptic peptides under study.

The data was analyzed against the mAb sequence using Spectrum Mill MS Proteomics Workbench. The result of such an analysis is shown in **Figure 3**. **Figure 4** shows the representative MS/MS spectrum with the assigned sequence for example heavy and light chain peptides.

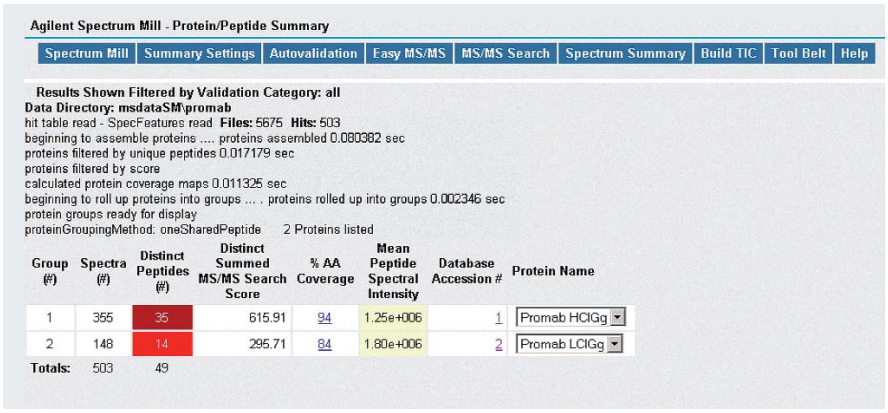
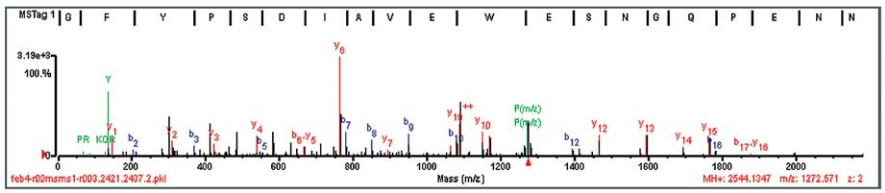


Figure 3: Result window for Spectrum Mill based database search for the mAb trypsin digest.

A



B

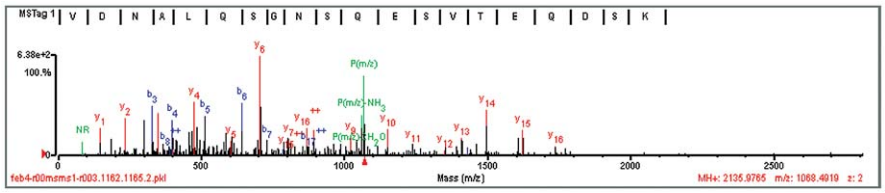


Figure 4: Representative MS/MS spectra of peptides from heavy chains (A) and light chains (B) with peptide sequences from Spectrum Mill MS Proteomics Workbench analysis.

Conclusions

The HPLC-Chip technology provides chromatographic peaks with narrow peak widths, which are important to generate high quality peptide fingerprints for large, complex proteins.

The highly accurate peptide masses determined by the Agilent 6520 Accurate-Mass Q-TOF LC/MS enables clear assignment of peptide peaks to the mAb sequence under study.

Data analysis using BioConfirm software helps with easy annotation and interpretation of the results.

The combination of HPLC-Chip technology with an Accurate-Mass Q-TOF mass spectrometer is a valuable tool for peptide mapping of small quantity biopharmaceuticals, providing analytical power that enhances peptide mapping studies.

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