APPLICATION NOTE

Antibody Fc Glycan Analysis by FabRICATOR® Digestion and LC-MS

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ABSTRACT

Using the FabRICATOR (IdeS) enzyme for antibody subunit analysis has become a standard analytical method for rapid characterization of monoclonal antibodies (mAbs). FabRICATOR digests IgG below the hinge, generating F(ab')2 and Fc/2 fragments. The unique specificity of the enzyme has led to the development of a range of analytical strategies for middle-level characterization of therapeutic mAbs and related products, such as antibody-drug conjugates (ADCs), bispecific antibodies and Fc-fusion proteins. The single FabRICATOR digestion site and the following accuracy of the mass profile have enabled detection of the antibody glycosylation profile and other critical quality attributes.

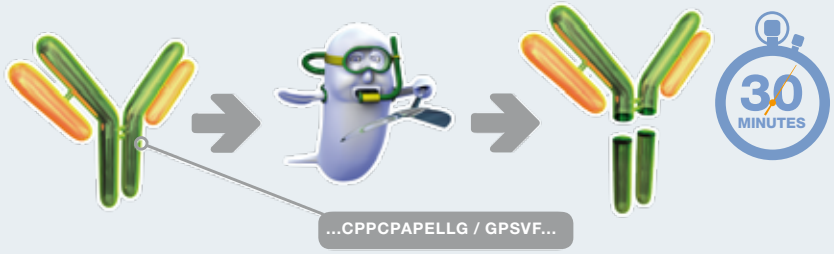
In this application note, the Fc glycosylation profile of the therapeutic antibody trastuzumab was analyzed using FabRICATOR in combination with LC-MS. The antibody was digested with FabRICATOR, denatured and reduced, and the resulting Fc/2, Fd' and light chain (LC) fragments were separated and analyzed by LC-MS. The short digestion time of FabRICATOR in combination with the ~25 kDa Fc/2 fragment, enabled a fast analysis of the glycan profile with monoisotopic resolution when analyzed by LC-MS. The presented workflow is robust and can be used to study not only glycosylation, but also critical quality attributes such as oxidation, glycation and sequence identity.

SUMMARY

- The Fc glycosylation profile of trastuzumab was determined with high accuracy compared to theoretical values
- FabRICATOR (IdeS) enables a rapid and easy platform method for IgG fingerprinting
- FabRICATOR specifically digests IgG below the hinge wihin 30 minutes
- Detailed methods for separation and mass spectrometry analysis at the middle-level are described

PRODUCT

FabRICATOR®



FabRICATOR is an IgG-specific cysteine protease that digests antibodies at a single amino acid site below the hinge region, generating a homogenous pool of F(ab')2 and Fc/2 fragments within 30 minutes. Neutral pH and no requirements for co-factors make the enzyme easy to use and enable platform analytical workflows based on FabRICATOR without the need for optimization. FabRICATOR is widely used in characterization, quality control, stability testing, production monitoring and clone selection of antibody-based therapeutics, such as mAbs, ADCs, biosimilars and Fc-fusion proteins.

- Human IgG1-4, Fc-fusion proteins, ADCs, mouse IgG2a and IgG3, IgG from monkey, rat, rabbit and sheep
- 30 min reaction
- No need for reducing agents or co-factors
- CPAPELLG / GPSVF (below the hinge)

INTRODUCTION

Recombinant mAbs for therapeutic use have been developed as effective drugs against various diseases, such as cancer and autoimmune diseases. A critical quality attribute of therapeutic antibodies is the glycosylation profile, since it may affect prominent features like stability and solubility as well as pharmacodynamic and pharmacokinetic properties. The glycosylation process is a part of the posttranslational modification of proteins and close monitoring of this attribute during the bioprocess development and the manufacturing is very important[1,2].

A majority of all commercial therapeutic antibodies are of class IgG and contain an N-glycan in the Fc domain. To measure the Fc glycosylation, a widely accepted method is to apply hydrophilic interaction liquid chromatography-ultra high-performance liquid chromatography (HILIC-UHPLC) on released N-glycans after being labeled with 2-aminobenzamide (2-AB)[1,2]. This methodology suffers from several hands-on steps and is time consuming.

An alternative method for glycan profile analysis has been suggested, involving direct analysis of the antibody Fc/2 fragment using LC-MS[2,3]. The cysteine protease FabRICATOR (IdeS) digests human IgG at a specific site below the hinge, generating a homogenous pool of Fc/2 and F(ab')2 fragments[4] (*Fig. 1*). The smaller fragment size of Fc/2 (~25 kDa) compared to the intact heavy chain (HC, ~50 kDa), allows for monoisotopic resolution of the glycan profile when analyzed with ESI-MS. Digestion using FabRICATOR is rapid, 30 minutes, and due to its specific activity, there is no risk of overdigestion that might otherwise result in heterogeneity of the antibody sample[2]. In an extensive comparison between methods for Fc glycosylation analysis, the middle-up analysis of Fc/2 glycans was found to be robust and to provide reliable results for the IgG glycoprofile[5].

This application note presents a workflow for evaluating the glycan profile of the therapeutic human IgG1 trastuzumab using FabRICATOR in combination with LC-MS analysis. The measured monoisotopic masses of Fc/2, LC and Fd' fragments are compared to theoretical data and the relative distribution of the different Fc glycoforms is calculated.

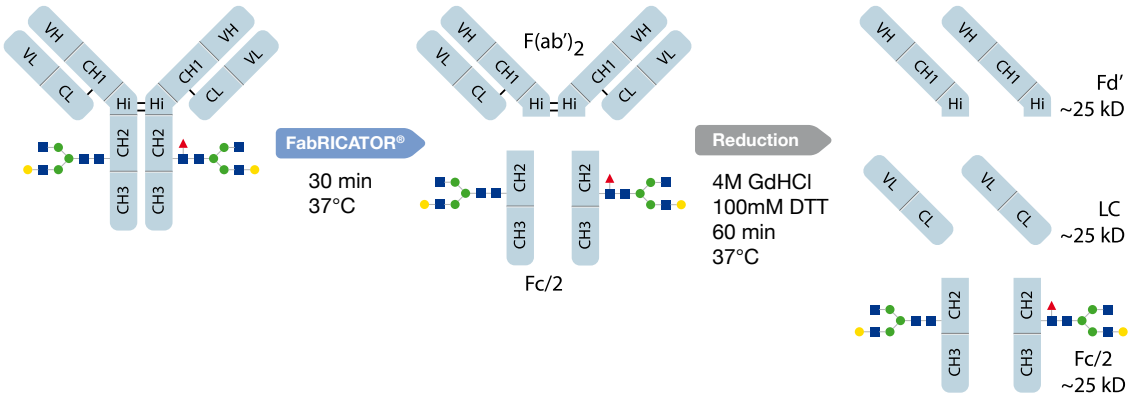


Figure 1. The IgG is digested with FabRICATOR followed by a denaturation and reduction step for LC-MS analysis.

MATERIALS & METHODS

Antibody Digestion using FabRICATOR

2000 units of FabRICATOR was reconstituted in 30 µl Milli-Q water (MQ) to a final concentration of 67 U/µl. To obtain a final ratio of 1 unit enzyme per 1 µg of antibody, 4 µl trastuzumab (25 mg/ml) and 1.5 µl FabRICATOR (67 U/µl), was added to 44.5 µl PBS to a final IgG concentration of 2 mg/ml. The sample was then incubated at 37°C for 30 minutes (for an overview of the workflow, see *Fig. 2*).

Figure 2. FabRICATOR was reconstituted in MQ before added to IgG in PBS for digestion.

Denaturation and Reduction of Antibody Subunits

6 µl of the FabRICATOR-digested IgG sample (2 mg/ml), was added to 10 µl 8M guanidine hydrochloride (GdHCl) and 4 µl 0.5 M DTT (fresh) to final concentrations of 0.6 mg/ml IgG, 4M GdHCl and 100 mM DTT. This prepared sample was then denatured and reduced by incubation at 37°C for 60 minutes prior to LC-MS analysis. An overview of the FabRICATOR sample preparation for LC-MS analysis is given in *Fig. 1*.

Analysis by Liquid Chromatography and Mass Spectrometry

The prepared IgG sample was analyzed using LC-MS. To avoid sample buffer exchange prior to MS analysis, a 13 minutes desalting step was added before an acetonitrile/water gradient was applied to a BEH C4 column from Waters according to Table 1 and Table 2. During the desalting step, the valve was switched to waste. The separated IgG fragments were then analyzed by ESI-Q-TOF MS on a Bruker Impact II instrument under conditions given in Table 3. The obtained TIC data were deconvoluted using the MaxEnt algorithm to determine masses. The theoretical masses were calculated from amino acid sequences obtained from the KEGG database (http://www.genome.jp/kegg/).

Table 1. Liquid Chromatography Setup

LC system	Agilent 1290 Infinity
Sample temp.	8°C
Flow rate	0.2 ml/min
Injection volume	1.7 µl
Sample load	1 µg
Column	Acquity UPLC Protein column, BEH C4, 300Å, 1.7 µm, 2.1 mm x 100 mm column (Waters)
Column temp.	50°C
Method time	42 minutes

Table 2. Liquid Chromatography Method

RP-LC solvent gradient		
Time (min)	Solvent A (%): 0.1% formic acid in MQ	Solvent B (%): 0.1% formic acid in 95% ACN: 5% MQ
0	90	10
13	90	10
14	75	25
26	54	36
29	10	90
32	10	90
32.01	90	10
42	90	10

Table 3. Mass Spectrometry Conditions

Instrument	Bruker Impact II
Analysis method	ESI-Q-TOF MS
Resolving power	50 000
Mass range	300 - 3000 m/z
ESI source voltage	4.5 kV
ESI source temp.	220°C
Nebulization gas pressure	1.8 Bar
Nebulization gas flow rate	8.0 l/min
Data analysis program	Compass DataAnalysis 2.0 (Bruker)
Deconvolution algorithm	MaxEnt

RESULTS & DISCUSSION

After denaturation and reduction, the FabRICATOR-digested trastuzumab fragments eluted as three protein peaks detected with UV 280 when separated by RP-LC (*Fig. 3*).

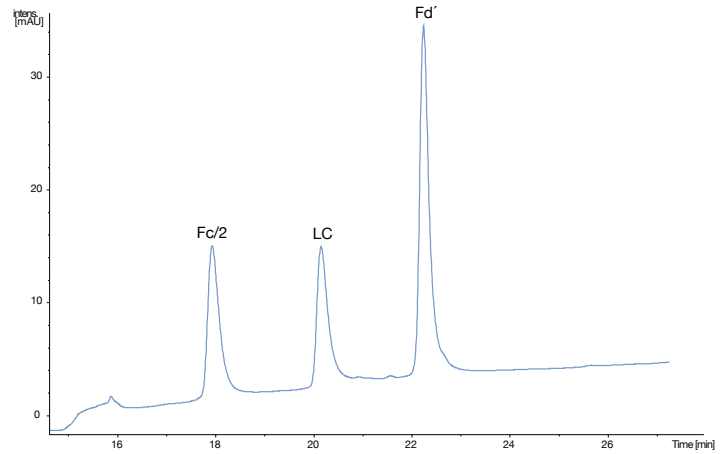


Figure 3. HPLC chromatogram (UV 280 signal) of FabRICATOR-digested trastuzumab after denaturation and reduction. The three fragments Fc/2, LC and Fd' are depicted in the chromatogram.

Mass determination of the three peaks with ESI-Q-TOF MS followed by analysis of the deconvoluted TIC data with the MaxEnt algorithm, confirmed that the mass of the peaks corresponded to theoretical values of the reduced forms of the Fc/2, Fd' and LC fragments, with a high mass accuracy (Δppm < 2; *Fig. 4-6* and Table 4). The monoisotopic masses of the four main trastuzumab Fc/2 glycoforms were determined and their relative intensities were calculated (*Fig. 4* and Table 5).

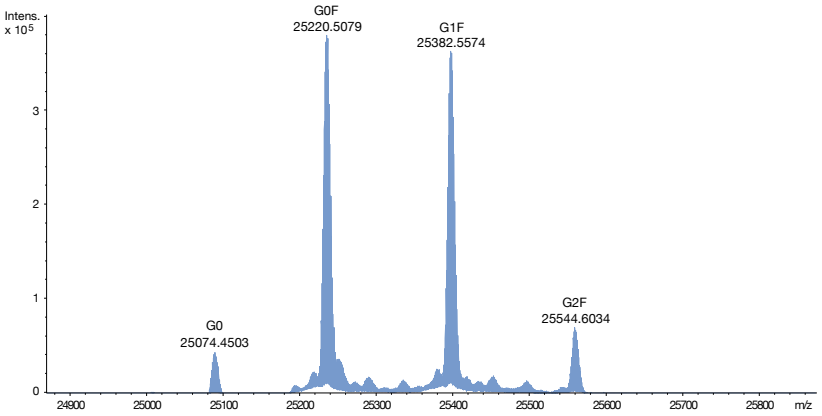


Figure 4. Deconvoluted mass spectrum of trastuzumab Fc/2 fragments after FabRICATOR digestion, denaturation and reduction. The different glycoforms of Fc/2 are depicted in the spectrum.

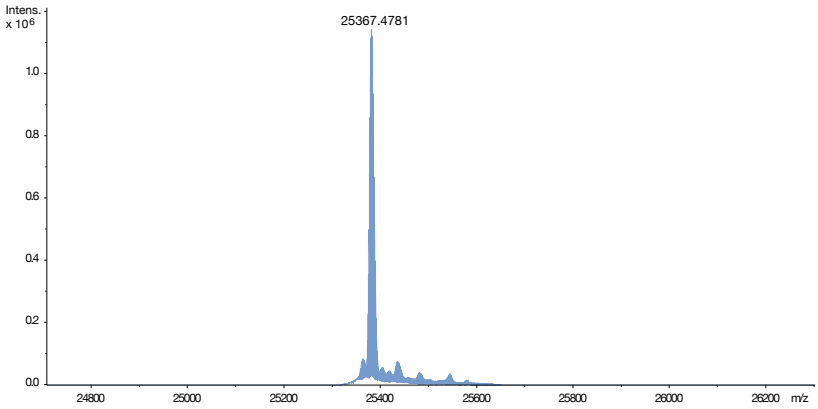


Figure 5. Deconvoluted mass spectrum of trastuzumab Fd' fragment after FabRICATOR digestion, denaturation and reduction.

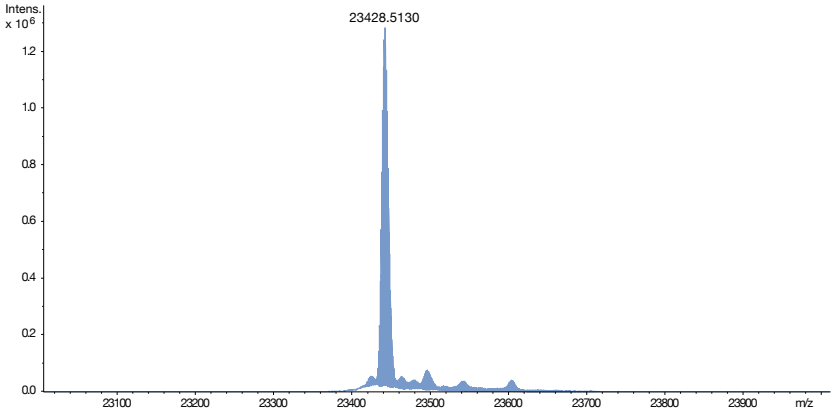


Figure 6. Deconvoluted mass spectrum of trastuzumab LC fragment after FabRICATOR digestion, denaturation and reduction.

Table 4. Mass Determination of Detected Peaks

Fragment	Detected Mass (Da)	Theoretical Mass (Da)	Mass Δ(ppm)
Fc/2 (240-449) + G0F	25220.5079	25220.4634	1.8
Fc/2 (240-449) + G1F	25382.5574	25382.5162	1.6
Fc/2 (240-449) + G2F	25544.6034	25544.5690	1.3
Fc/2 (240-449) + G0	25074.4503	25074.4055	1.8
Fd' (1-239)	25367.4781	25367.5174	-1.5
LC	23428.5130	23428.5238	-0.5

Table 5. Relative Intensities of Fc/2 Glycoforms of Trastuzumab

Fc/2 Glycoforms	Relative intensity (%)
G0	4.8
G0F	44.7
G1F	42.7
G2F	7.9

The relative glycoform distribution was found to correlate well with data from other methods for glycan analysis, such as HILIC-UHPLC separation of released and 2-AB labeled glycans as well as LC-MS analysis of reduced, intact antibody[6,7]. The sample preparation prior to LC-MS analysis included digestion of the antibody using FabRICATOR for 30 minutes, followed by denaturation and reduction of the antibody fragments for 60 minutes. Buffer exchange prior to MS analysis was avoided by incorporating a desalting step in the HPLC gradient. The denaturation and reduction steps can be excluded if there is no interest in the Fd' and LC fragments.

In conclusion, we here present a fast and easy workflow to determine the glycan composition of therapeutic antibodies, and the same workflow can be utilized to analyze several critical quality antibody attributes[2]. This method is useful within both development and production of mAbs, for example to control the glycan profile during process development and to assure batch-to-batch consistency.

ABBREVIATIONS

ACN	acetonitrile	**LC**	light chain
ADC	antibody-drug conjugate	**LC-MS**	liquid chromatography - mass spectrometry
DTT	dithiothreitol		
ESI-MS	electrospray ionization mass spectrometry	**mAb**	monoclonal antibody
		MQ	Milli-Q water
GdHCl	guanidine hydrochloride	**RP-LC**	reversed phase liquid chromatography
HC	heavy chain		
HILIC-UHPLC	hydrophilic interaction liquid chromatography-ultra-high performance liquid chromatography	**TIC**	total ion chromatogram
HPLC	high performance liquid chromatography		

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