# Characterization of Monoclonal Antibodies Oxidation Variants Using Middle-down Approach and Hydrogen/Deuterium Exchange Mass Spectrometry

Poster Note 21224

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## Overview

**Purpose:** To achieve high resolution separation of mAb fragments and oxidized fragments.

**Methods:** Reverse phase separation of mAb and mAb fragments using Thermo Scientific™ MAbPac™ RP column coupled with Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap mass spectrometer.

**Results:** LC, HC, Fc, Fab, scFc and F(ab′)$_2$ are successfully separated using a 10-min gradient. Mass of oxidized HC is fully resolved from non-oxidized HC.

## Introduction

The monoclonal antibody (mAb) therapeutics market is growing at a rapid rate owing to increased demand for targeted treatments. Therapeutic mAbs, such as rituximab, trastuzumab, infliximab, and bevacizumab, are mostly produced from mammalian cells. These biological products are heterogeneous due to post-translational modifications. Additional modifications such as oxidation can be introduced during the manufacturing process. A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing processes.

There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of mAbs using high resolution mass spectrometers. The most commonly employed LC/MS method is to desalt mAbs via reverse phase liquid chromatography and perform an MS analysis. Further MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), Fc, and Fab can quickly reveal the location as well as nature of the modification.

In the current study, we are presenting a fast separation method for mAb fragments rituximab, trastuzumab, infliximab, and bevacizumab using a novel supermacroporous reversed phase (SMP RP) column. The mAb fragments were then generated by subsequent DTT reduction, papain digestion, or IdeS protease digestion. Baseline separation of light chain and heavy chain, Fc and Fab fragments, scFc and F(ab')$_2$ was achieved in all cases using a 10-min gradient with water/acetonitrile/FA/TFA mobile phases. Using an orbitrap mass spectrometer accurate masses of mAb fragments were measured and the presence of oxidation variants was detected. The fast LC/MS approach described here can be generally applicable to mAb variant characterization.

## Methods

### Chemicals and reagents

Papain was purchased from Sigma-Aldrich©. IdeS Protease was purchased from Genovis. Therapeutics monoclonal antibodies rituximab, trastuzumab, infliximab, and bevacizumab were from the original manufacturers.

### Column

MAbPac RP 4 µm, 3 × 50 mm, P/N 088645

### LC instruments

Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with: SRD-3400 Solvent racks with degasser, HPG-3400RS Biocompatible Binary Rapid Separation Pump , WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler, TCC-3000RS Rapid Separation Thermostatted Column Compartment , VWD-3400RS Rapid Separation Variable Wavelength Detector

### Mobile phases:

Mobile phase A: 0.1% FA + 0.02% TFA + 99.88 % $H_2O$

Mobile phase B: 0.1% FA + 0.02% TFA + 90% MeCN + 9.88% $H_2O$

### Reduction:

Reduction of inter-chain disulfides in a mAb (4 mg/mL) was achieved by incubation of mAb with 20 mM DTT at 37 °C for 30 min.

### Papain digestion:

The digestion was carried out by incubating mAb (2 mg/mL) with papain (0.04 mg/ml) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA and 5 mM Cysteine buffer at 37 °C for 4 hours.

### IdeS digestion:

IdeS protease was added at 1 unit enzyme per 1 µg of mAb ratio. The digestion was carried out in 50 mM sodium phosphate, 150 mM NaCl (pH 6.6) buffer at 37 °C for 30 min.

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### Preparation of $H_2O_2$ oxidized mAb

Dilute the mAb solution (5 mg/mL) in half with the 2X oxidation buffer (360 mM sodium chloride, 10 mM sodium acetate, pH 5.0). Then add $H_2O_2$ to a final concentration of 0.01% (v/v) and incubate the sample for 24h at room temperature.

### MS conditions for mAb and mAb Fragment Analysis

The Q Exactive Plus Orbitrap mass spectrometer was used for this study. Intact mAb or mAb fragments were analyzed by ESI-MS. HESI probe was used. The resolution was set at 17.5 k.

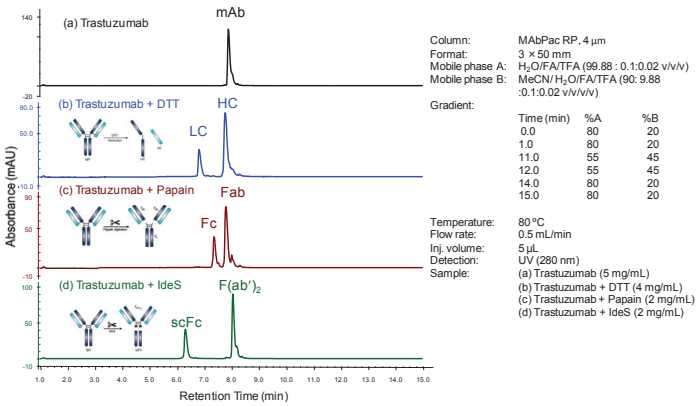
### Hydrogen/Deuterium Exchange Mass Spectrometry

Both non-oxidized and oxidized mAb were diluted (1 to 9 ratio) with labeling buffer and incubated for multiple time points. The samples were then quenched with 4M guanidine, 200 mM citric acid (pH 2.7) at 0.5 °C and subject to online pepsin digest at 8 °C for three minutes at 50 µL/min flow rate in a fully automated manner using H/D-X PAL (LEAP Technologies). The digested peptides were injected into a PepMap trapping column washed for one minute and eluted to a Hypersil GOLD C18 reverse phase column. An UltiMate 3000 nano pump system was employed to separate the digested peptides with 5% to 40% mobile phase B in 6 minutes gradient at flow rate of 40 µL/min. The separated peptides MS analysis was performed with Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer at 60K resolution.

## Results

Monoclonal antibodies are heterogeneous. Comprehensive analysis of mAb post-translational modifications, such as deamidation, C-terminal lysine truncation, *N*-terminal pyroglutamation, methionine (Met) oxidation, and glycosylation, requires complete digestion of the mAbs and sequencing of all the peptides. However, "peptide mapping" is time consuming. A simpler and direct way to analyze the mAb variants and locate the modifications is to measure mAb fragments. Light chain (LC) and heavy chain (HC) are generated by the reduction of mAb, Fc and Fab fragments are generated by papain digestion. Single chain Fc (scFc) and $F(ab')_2$ fragments are generated by IdeS digestion. Figure 1 shows the analysis of trastuzumab intact molecule and trastuzumab fragments. LC and HC (Figure 1b), Fc and Fab (Figure 1c), scFc and $F(ab')_2$ (Figure 1d) are baseline separated using a MAbPac RP column with a 10-min gradient. Similar experiments have been carried out for rituximab, infliximab, and bevacizumab. In all cases, mAb fragments have been successfully separated (data not shown).

**FIGURE 1. Analysis of trastuzumab and fragments using MAbPac RP.** (a) trastuzumab; (b) trastuzumab LC and HC; (c) trastuzumab Fc and Fab fragments; (d) trastuzumab scFc and $F(ab')_2$ fragments.




Met oxidation is one of the critical quality attributes required to be closely monitored. The two Met residues in the CH2-CH3 domain interface of recombinant humanized and fully human IgG1 antibodies were found susceptible to oxidation [1]. It is desirable to monitor the progress of the Met oxidation without complete digestion of mAb. A workflow was designed to first reduce mAb and then further digest it with IdES resulting in smaller (25 kDa) fragments (Figure 2). Figure 3a shows that oxidized HC and non-oxidized HC can be barely separated chromatographically. However, the high resolution orbitrap instrument can clearly resolve the oxidized (Figure 3b) and non-oxidized HC (Figure 3c) at *m/z* 1633.54 and 1633.06 respectively. Further digestion of the HC by IdeS resulted in two smaller fragments: scFc and Fd′. Figure 4a shows the baseline separation of scFc, LC, and Fd′. In addition, oxidized and non-oxidized scFc fragments are better separated than the oxidized and non-oxidized HC. The +10 charge state of the oxidized scFc and non-oxidized scFc are shown in Figure 4b (at *m/z* 2525.60) and in Figure 4c (at *m/z* 2524.08).

**FIGURE 2. mAb reduction and IdeS digestion flowchart.**

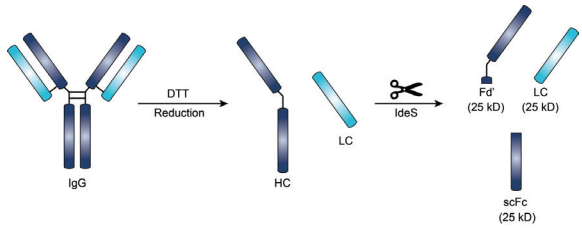
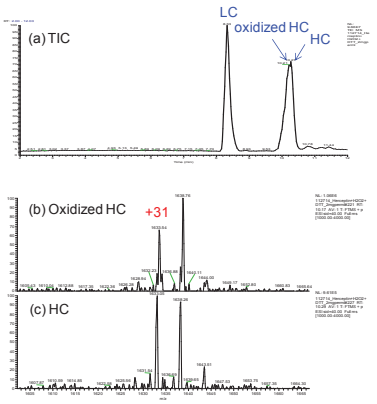


**FIGURE 3. LC/MS analysis of trastuzumab LC and HC.** (a) Total ion current (TIC); (b) mass spectrum of oxidized HC; (c) mass spectrum of non-oxidized HC.

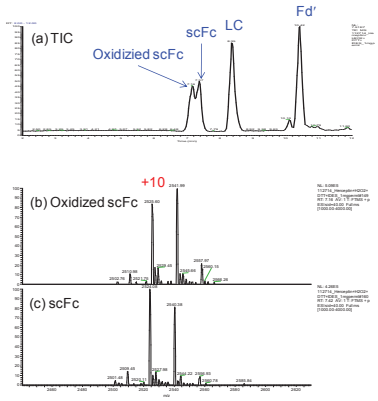



| | |
|---|---|
| Column: | MAbPac RP, 4 µm |
| Format: | 3 × 50 mm |
| Mobile phase A: | $H_2O$/FA/TFA (99.88 : 0.1:0.02 v/v/v) |
| Mobile phase B: | MeCN/ $H_2O$/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v) |
| Gradient: | |

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 75 | 25 |
| 1.0 | 75 | 25 |
| 11.0 | 63 | 37 |
| 12.0 | 63 | 37 |
| 14.0 | 75 | 25 |
| 15.0 | 75 | 25 |

| | |
|---|---|
| Temperature: | 80 °C |
| Flow rate: | 0.5 mL/min |
| Inj. volume: | 2 µL |
| MS Detection: | positive-ion mode |
| Mass spec: | Q Exactive™ Plus |
| Sample: | oxidized trastuzumab, reduced by DTT (2 mg/mL) |

**FIGURE 4. LC/MS analysis of trastuzumab scFc, LC, and Fd′.** (a) Total ion current (TIC); (b) mass spectrum of oxidized scFc; (c) mass spectrum of non-oxidized scFc.



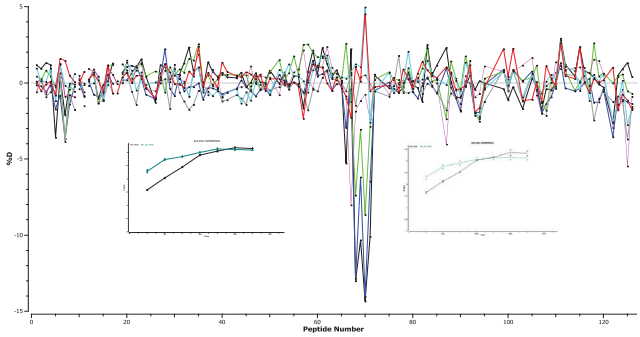

| | |
|---|---|
| Column: | MAbPac RP, 4 µm |
| Format: | 3 × 50 mm |
| Mobile phase A: | $H_2O$/FA/TFA (99.88 : 0.1:0.02 v/v/v) |
| Mobile phase B: | MeCN/ $H_2O$/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v) |
| Gradient: | |

| Time (min) | %A | %B |
|---|---|---|
| 0.0 | 75 | 25 |
| 1.0 | 75 | 25 |
| 11.0 | 63 | 37 |
| 12.0 | 63 | 37 |
| 14.0 | 75 | 25 |
| 15.0 | 75 | 25 |

| | |
|---|---|
| Temperature: | 80 °C |
| Flow rate: | 0.5 mL/min |
| Inj. volume: | 2 µL |
| MS Detection: | positive-ion mode |
| Mass spec: | Q Exactive™ Plus |
| Sample: | Oxidized trastuzumab, reduced by DTT and digested by IdeS (1 mg/mL) |

In Figure 5, heavy chain deuterium uptake difference between trastuzumab and its oxidized variants is plotted vs. peptide number. The data were obtained with HDExaminer from MS full scan at various deuterium exchange time points. At most regions the difference is minimal, except at the specific region where in the vicinity of methionine (residue 255). This is the amino acid that is oxidized. The inserts of Figure 5 show the different kinetic behavior of deuterium uptake of trastuzumab and its oxidized forms; after oxidation, the deuterium uptake is faster. Structurally, it is more sterically accessible for solvent exchange when methionine's $SCH_3$ terminal is oxidized to $SOCH_3$ or $SO_2CH_3$.

**FIGURE 5. Trastuzumab versus trastuzumab oxidation variants heavy chain deuterium uptake residual plot.** Inserts are the specific peptides (244-254 FLFPPKPKDTL and 245-254 LFPPKPKDTL) deuterium uptake plots of trastuzumab versus its oxidation variants .




## Conclusions

- mAb LC, HC, Fc, Fab, scFc and F(ab′)$_2$ fragments are successfully separated using MAbPac RP column and a 10-min gradient.
- Mass of oxidized HC is fully resolved from non-oxidized HC using Q Exactive Plus mass spectrometer.
- The H/D exchange workflow pinpoints the subtle but significant changes in the methionine region.

## References


1. Liu H., Gaza-Bulseco G., and Zhou L. Mass Spectrometry Analysis of Photo-InducedMethionine Oxidation of a Recombinant Human Monoclonal Antibody, J. Am. Soc. Mass. Spectrom., 2009, 20, 525-528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