Fast Analysis of Therapeutic Monoclonal Antibody Fragments Using a Supermacroporous, Reversed-Phase Chromatography Column

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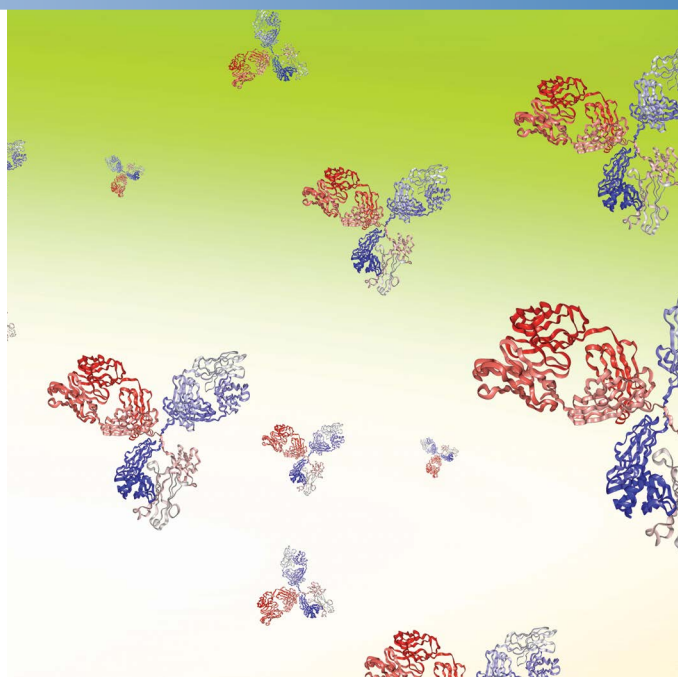
Application Note 21200

Key Words
MAbPac RP, rituximab, trastuzumab, infliximab, bevacizumab

Goal
To describe a high-resolution, high-speed separation of monoclonal antibody (mAb) fragments using a supermacroporous, reversed-phase column. Baseline separations of light chain (LC) and heavy chain (HC), crystallizable fragment (Fc) and antigen-binding fragment (Fab), single chain Fc (scFc) and $F(ab')_2$ were achieved for rituximab, trastuzumab, infliximab, and bevacizumab using a 10 minute gradient with water/acetonitrile/formic acid/trifluoroacetic acid mobile phases.



Introduction

A comprehensive characterization of mAb purity, aggregates, and variants is required for the final biopharmaceutical product approval and subsequent manufacturing process. We chose therapeutic mAbs (rituximab, trastuzumab, infliximab, and bevacizumab), produced mostly by mammalian cells, to study the reversed-phase chromatographic behavior of intact mAbs as well as of their fragments. These biological products are heterogeneous due to post-translational modifications.[1] Additional modifications, such as oxidation, could be introduced during the manufacturing process,[2] and the results of this analysis are also included.

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 (MS). The most commonly employed LC-MS method is to analyze mAbs via reversed-phase liquid chromatography coupling with mass spectrometry detection. Further MS analysis of mAb fragments such as light chain (LC), heavy chain (HC), crystallizable fragment (Fc), and antigen binding fragment (Fab) can quickly reveal the location as well as nature of the modification.[3]

Here, we present a fast separation method for mAb fragments from rituximab, trastuzumab, infliximab, and bevacizumab using a novel supermacroporous, reversed-phase column. Baseline separations of LC and HC, Fc and Fab, single chain Fc (scFc) and $F(ab')_2$ fragments (two antigen-binding Fab portions linked together by disulfide bonds) were achieved in all cases using a 10 min gradient with water/acetonitrile/formic acid/trifluoroacetic acid mobile phase. The results demonstrate the general applicability of Thermo Scientific™ MAbPac™ RP column for mAb analysis.

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Experimental

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Fisher Chemical™ Optima™ LC/MS acetonitrile (MeCN) [C_2H_3N; P/N A955-1]
- Fisher Chemical Optima LC/MS formic acid (FA) [CH_2O_2; P/N A117-1AMP]
- Fisher Chemical Optima LC/MS trifluoroacetic acid (TFA) [$C_2HF_3O_2$; P/N A116-50]
- Dithiothreitol (DTT) and papain, purchased from Sigma-Aldrich®
- FabRICATOR® (IdeS protease), purchased from Genovis
- Rituximab, trastuzumab, infliximab, and bevacizumab were from the original manufacturers

Sample Handling Equipment

Thermo Scientific™ Polypropylene, 0.3 mL vials (P/N 055428)

Sample Preparation

Reduction

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

Papain digestion

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.

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system equipped with:		
	SRD-3400 Solvent Racks with Degasser (P/N 5035.9245)		
	HPG-3400RS Biocompatible Binary Rapid Separation Pump (P/N 5040.0046)		
	WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler (P/N 5841.0020)		
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.0000)		
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) equipped with a semi-micro flow cell PEEK, 2.5 µL volume, 7 mm path length		
Column	MAbPac RP, 4 µm, 3.0 × 50 mm (P/N 088645)		
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	80	20
	1.0	80	20
	11.0	55	45
	12.0	55	45
	14.0	80	20
	15.0	80	20
Flow rate	0.5 mL/min		
Column temperature	80 °C		
UV detector wavelength	280 nm		
Injection volume	5 µL		
Samples	Rituximab, 5 mg/mL; trastuzumab, 5 mg/mL; infliximab, 5 mg/mL; bevacizumab, 5 mg/mL		

Software

The Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System was used for data acquisition and analysis.

Results and Discussion

The MAbPac RP column is packed with supermacroporous polymer resin. Its large pore size (~ 1500 Å) is suitable for separation of large proteins, such as 150 kDa mAb and its 25, 50, and 100 kDa fragments. In addition, mAb analysis by reversed-phase liquid chromatography requires high temperatures such as 70 to 80 °C to reduce the strength of secondary interactions between mAb and the stationary phase.[4] The polymeric nature of the MAbPac RP column provides both pH and temperature stability, making it an ideal column for mAb analysis.

Monoclonal antibodies are heterogeneous (Figure 1). 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.

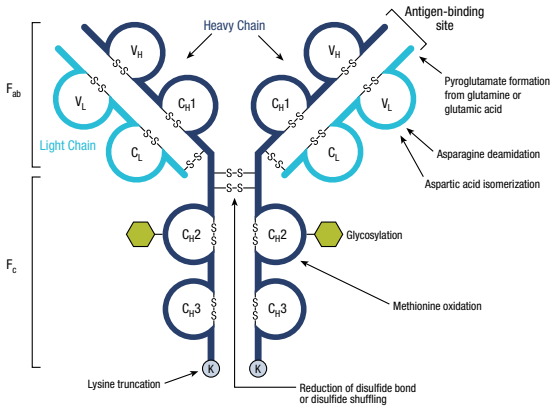


Figure 1. Structure of IgG and typical forms of heterogeneity.

However, peptide mapping can be time consuming. A simple and direct way to analyze mAb variants and locate the modification sites is to measure mAb fragments.[3] Light chains (LC) and heavy chains (HC) are generated by the reduction of mAbs with DTT (Figure 2a). Fc and Fab fragments are generated by papain digestion (Figure 2b). Single chain Fc (scFc) and F(ab')2 fragments are generated by IdeS digestion (Figure 2c).

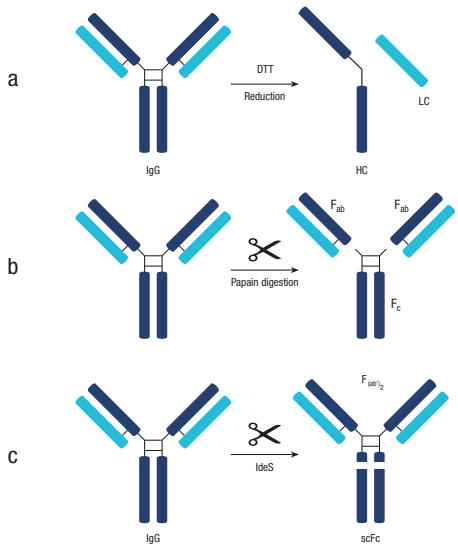
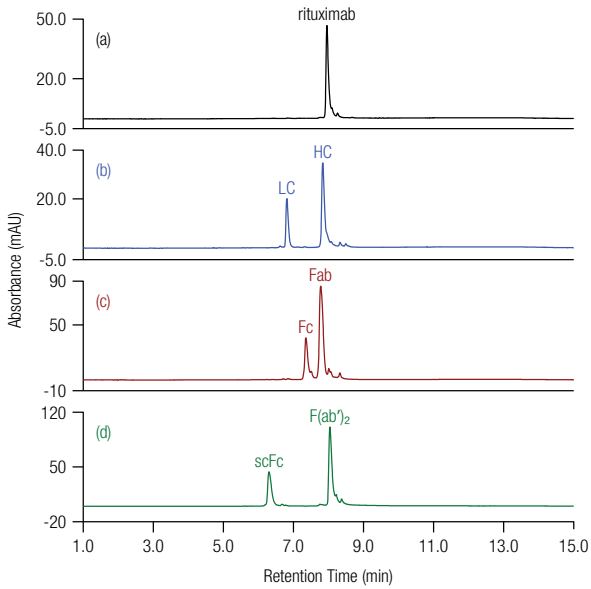


Figure 2. Monoclonal antibody fragments generation flow chart. (a) Reduction; (b) Papain digestion; (c) IdeS digestion.

Rituximab (Figure 3a), trastuzumab (Figure 4a), infliximab (Figure 5a), and bevacizumab (Figure 6a) were analyzed on an MAbPac RP column using a 10 minute gradient. In all cases, in addition to the main mAb peak, minor variant peaks eluting at a later retention time were observed. DTT reduction of mAb produces two copies of LC (25 kDa) and two copies of HC (50 kDa). The analysis of LC and HC of each mAb (Figures 3b, 4b, 5b, and 6b) showed two major peaks, plus small variant peaks eluting after HC. The peak area ratio between LC and HC is approximately 1:2. The degree of separation varied, with rituximab LC and HC giving the best resolution, and infliximab LC and HC the least.



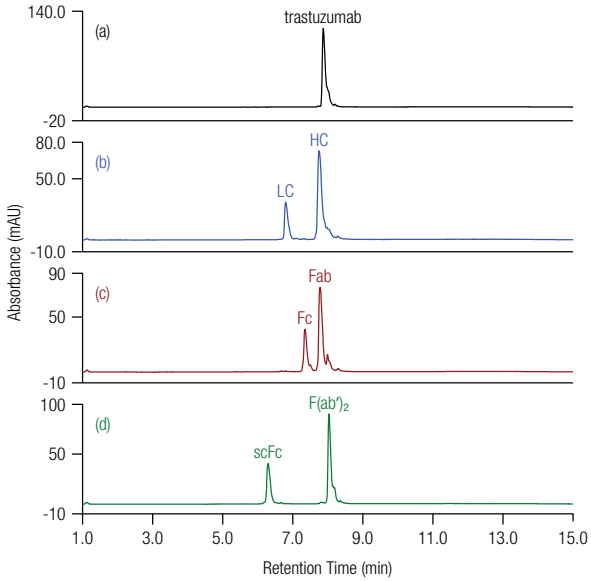
Column:	**MAbPac RP,** 4 µm
Format:	3 × 50 mm
Mobile Phase A:	H_2O/FA/TFA (99.88 : 0.1:0.02 v/v/v)
Mobile Phase B:	MeCN/ H_2O/FA/TFA (90: 9.88 :0.1:0.02 v/v/v/v)
Gradient:	

Time (min)	%A	%B
0.0	80	20
1.0	80	20
11.0	55	45
12.0	55	45
14.0	80	20
15.0	80	20

Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) rituximab (5 mg/mL) (b) rituximab + DTT (4 mg/mL) (c) rituximab + Papain (2 mg/mL) (d) rituximab + IdeS (2 mg/mL)

Figure 3. Analysis of rituximab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and $F(ab')_2$ fragments.

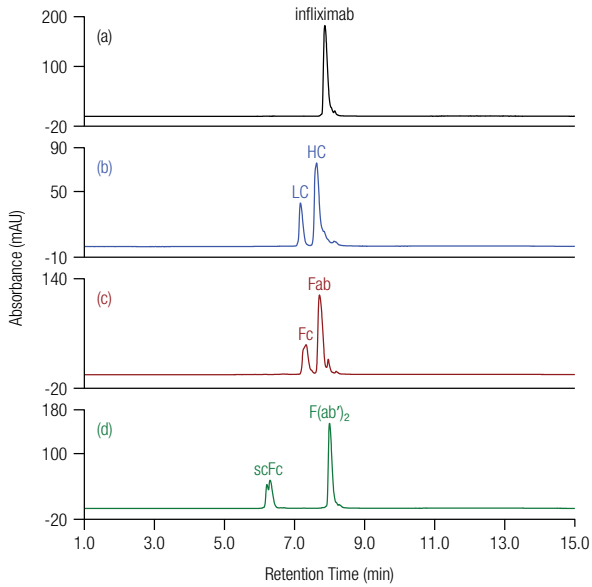
Papain digestion produces one copy of Fc (50 kDa) and two copies of Fab (50 kDa). The separation of Fc and Fab of each mAb (Figures 3c, 4c, 5c, and 6c) showed two major peaks, plus small variant peaks eluting after both Fc and Fab peaks. The peak area ratio between Fc and Fab is approximately 1:2. In the case of bevacizumab, we observed peaks co-eluting in the Fab region. Further MS analysis identified two polypeptide chains with the later eluting peak corresponding to the bevacizumab Fab, based on the molecular weight calculation. The earlier eluting peak has a smaller molecular weight comparing to the Fab (data not shown).



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 80 20 1.0 80 20 11.0 55 45 12.0 55 45 14.0 80 20 15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) trastuzumab (5 mg/mL) (b) trastuzumab + DTT (4 mg/mL) (c) trastuzumab + Papain (2 mg/mL) (d) trastuzumab + IdeS (2 mg/mL)

Figure 4. Analysis of trastuzumab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments.

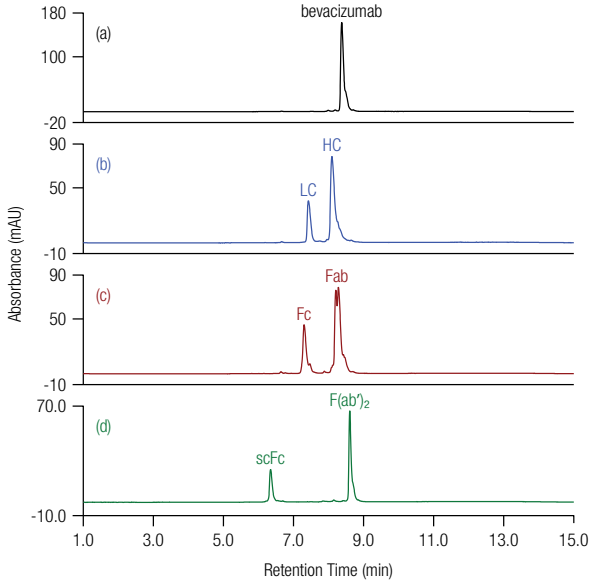
IdeS digestion produces two copies of scFc (25 kDa) and one copy of F(ab')$_2$ (100 kDa). The analysis of scFc and F(ab')$_2$ of each mAb (Figures 3d, 4d, 5d, and 6d) showed two major peaks, plus small variant peaks eluting after the F(ab')$_2$ peak. In the case of infliximab (Figure 5d), we observed two co-eluting scFc peaks. Further MS analysis revealed that the early eluting peak has one c-terminal lysine while the later eluting one does not have c-terminal lysine (data not shown). The chromatogram of infliximab Fc (Figure 5c) showed a broad peak. We hypothesize that this is due to co-eluting charge-variant species containing zero lysine, one C-terminal lysine, and two C-terminal lysines.



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 80 20 1.0 80 20 11.0 55 45 12.0 55 45 14.0 80 20 15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) infliximab (5 mg/mL) (b) infliximab + DTT (4 mg/mL) (c) infliximab + Papain (2 mg/mL) (d) infliximab + IdeS (2 mg/mL)

Figute 5. Analysis of infliximab and and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments.

The mobile phase used in this study contained 0.1% FA and 0.02% TFA. The lower TFA content resulted in a more MS-compatible eluent. Alternative mobile phases containing higher concentrations of TFA (0.1%) would result in better separation of mAb fragments.



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 80 20
	1.0 80 20
	11.0 55 45
	12.0 55 45
	14.0 80 20
	15.0 80 20
Flow Rate:	0.5 mL/min
Inj. Volume:	5 µL
Temp.:	80 °C
Detection:	UV (280 nm)
Sample:	(a) bevacizumab (5 mg/mL)
	(b) bevacizumab + DTT (4 mg/mL)
	(c) bevacizumab + Papain (2 mg/mL)
	(d) bevacizumab + IdeS (2 mg/mL)

Figure 6. Analysis of bevacizumab and fragments using MAbPac RP. (a) mAb; (b) LC and HC; (c) Fc and Fab fragments; (d) scFc and F(ab')$_2$ fragments

Conclusion

- The MAbPac RP column provides the ideal solution for separation of mAbs and related variants.
- The MAbPac RP column baseline separates reduction products: LC and HC.
- The MAbPac RP column baseline separates papain digest products: Fc and Fab.
- The MAbPac RP column baseline separates IdeS digest products: scFc and F(ab')$_2$.

References

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