Analysis of Monoclonal Antibody Oxidation Using Hydrophobic Interaction Chromatography

Atis Chakrabarti, Ph.D.
Tosoh Bioscience LLC

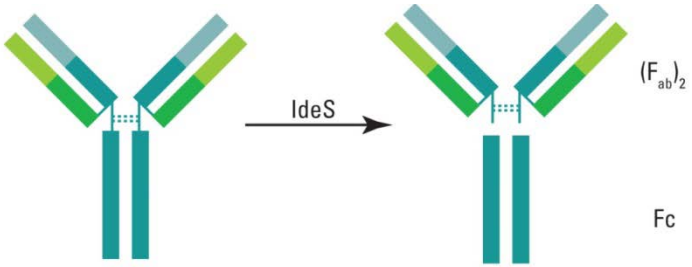
Introduction

- Oxidation of methionine or tryptophan residues has been associated with a decrease or elimination of biological activity of monoclonal antibody (mAb) biotherapeutics. Therefore it is crucial to monitor oxidation in order to confirm the stability and clinical efficacy of therapeutic mAbs.
- Incubation of a mAb with oxidation agents such as hydrogen peroxide (H_2O_2) or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is generally accepted as a simulation of the chemical degradation and oxidative stress that a mAb may experience during storage and delivery.
- Because oxidation of the mAb results in conformational changes that impact the overall surface hydrophobicity of the molecule, hydrophobic interaction chromatography (HIC) can be used to characterize the oxidized mAbs under nondenaturing conditions. Additionally, HIC can be used to resolve the products of partial digestion of mAbs. This approach, paired with forced oxidation, may reveal more information about the nature of the oxidation that is observed on the intact mAb (Figure 1).
- Here we report the use of a 4.6 mm ID x 3.5 cm, 2.5 µm, analytical HIC column based on a nonporous polymethacrylate base material bonded with a butyl phase chemistry for this study.

Fig. 1: Mechanism of Papain and IdeS Fragmentation

Schematic diagram of fragmentation of a mAb by IdeS

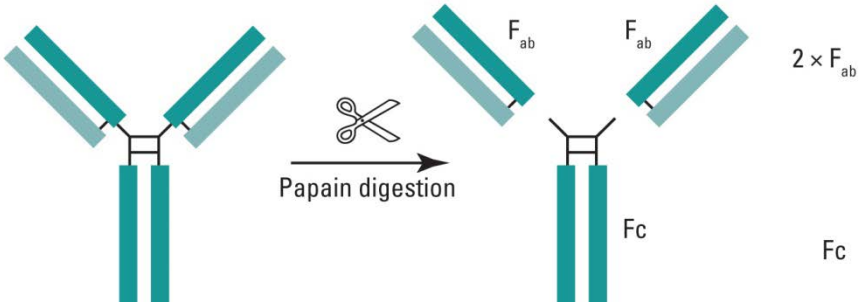
(Ref: mAbs 2014, 6:4, 879–893)



IgG_1 Hinge sequence: S-C-D-K-T-H-T-C-P-P-C-P-A-P-E-L-L-G-G-P-S-V

IdeS or IdeZ cleavage site

Schematic diagram of fragmentation of a mAb by papain



Materials and Methods

Chemicals and Reagents

- Deionized (DI) water - Barnstead 18.2 MΩ-cm resistivity
- Isopropanol - JT Baker 9095-33, Lot#L42303
- Sodium phosphate monobasic Anhydrous (KH_2PO_4·H2O) - VWR 0781, Lot# 0727C330 and Dibasic anhydrous (Na_2HPO_4)
- Ammonium sulfate $(NH_4)_2SO_4$) - VWR 0191, Lot# 0677C015
- Ethylenediaminetetraacetic acid (EDTA) tetra-sodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8$ $2H_2O$) - EMD EX 0550-5 Lot# 45350742
- L-cysteine - Fisher Scientific BP376, Lot# 122363
- Hydrogen peroxide (H_2O_2) 30% - VWR BDH7690 Lot#18A175006
- AAPH [2,2'-azobis(2-amidinopropane)] - Acros Organics 401560250 Lot# A0390287
- IdeS - Promega V7511 Lot# 00000302593
- Papain, lyophilized (≥10 units/mg protein) - Sigma P4762, Lot#107H70151

Materials and Methods (cont.)

- **Oxidation of mAb using 0.1% H_2O_2 :** 20 µL of mAb solution was mixed with 50 µL of 2x oxidation buffer. H_2O_2 solution in water was added to a final concentration of 0.01% (v/v) and the sample was incubated for 24 h at room temperature.
- **Preparation of 2x oxidation buffer:** 100 mL solution containing 360 mmol/L sodium chloride and 10 mmol/L sodium acetate was made in DI water. The pH was adjusted to 5.0 with 10 N HCl.
- **Oxidation of mAb using AAPH:** 20 µL of mAb solution was mixed with 50 µL of 2x oxidation buffer. Then AAPH solution (10 mmol/L) in water was added to a final concentration of 1 mmol/L. The mixture was incubated the sample for 24 h at 40 ºC.

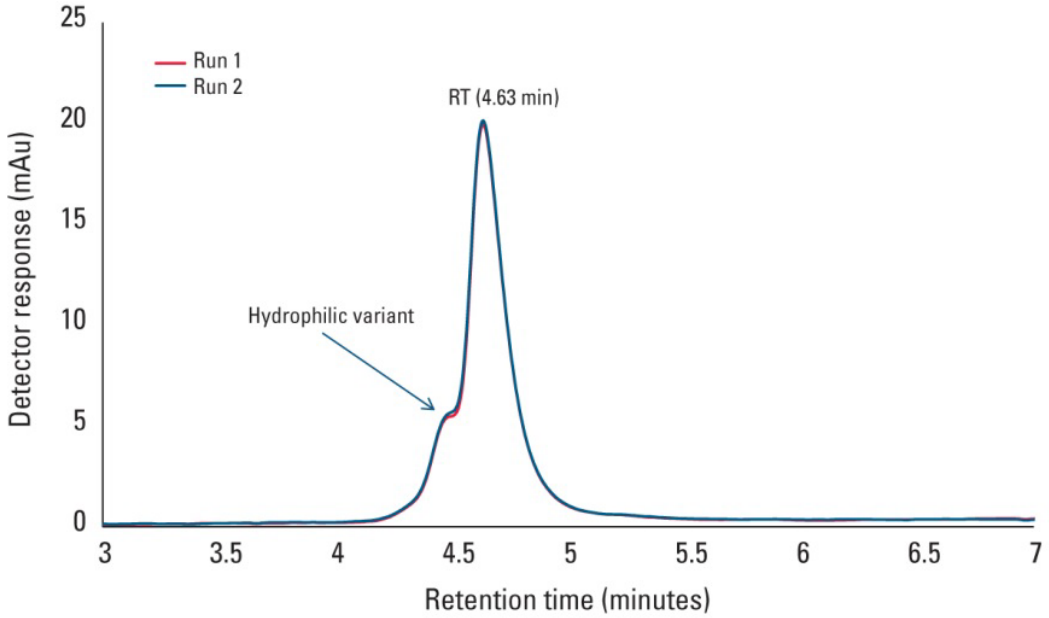
Materials and Methods (cont.)

- **2x digestion buffer:** 0.485 g of Tris, 0.060 g of EDTA, and 0.024 g of L-cysteine was dissolved in 15 mL of DI water. The pH was adjusted to 7.6 using HCl following the final volume adjustment to 20 mL with DI water.
- **Digestion of mAb using IdeS:** 20 µL of mAb solution was mixed with 50 µL of 2x digestion buffer. 1 unit of IdeS Protease per 1 µg of IgG was added. The sample was incubated at 37 ºC for 30 minutes.
- **Digestion of mAb using papain:** 2 mg/mL papain solution was prepared in DI water. 20 µL of mAb was mixed with 50 µL of 2x digestion buffer, 26 µL of DI water and 4 µL of Papain solution in water (2 mg/mL). The mixture was incubated in water bath at 37 ºC for 4 hours.

Materials and Methods (cont.)

Column:	TSKgel Butyl-NPR, 2.5 µm, non-porous, 4.6 mm ID x 3.5 cm
Instrument:	Agilent 1100
Mobile Phase:	**A**: 2 mol/L ammonium sulfate + 100 mmol/L phosphate buffer, pH 7.0
	B: 100 mmol/L phosphate buffer, pH 7.0
Gradient:	as indicated
Flow Rate:	as indicated
Detection:	UV @ 280 nm
Injection vol.:	5 µL
Sample:	NIST mAb (10 mg/mL)

Fig. 2: HIC Analysis of Intact NIST mAb using a TSKgel Butyl-NPR Column



Instrument: Agilent 1100

Mobile phase: **A**: 2 mol/L ammonium sulfate + 100 mmol/L phosphate buffer, pH 7.0
B: 100 mmol/L phosphate buffer, pH 7.0

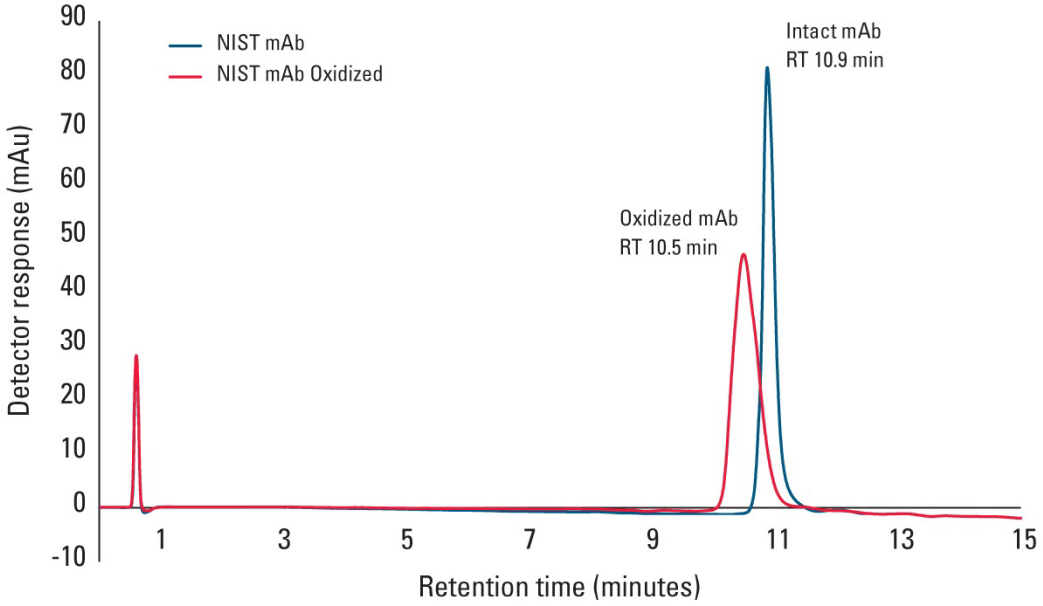
Gradient:

Time	%A	%B
0	60	40
15	0	100
20	0	100
20.1	60	40

Flow Rate: 1 mL/min
Detection: UV @ 280 nm
Injection vol.: 5 µL
Sample: NIST mAb (10 mg/mL)

A hydrophilic variant of NIST mAb was identified using a TSKgel Butyl-NPR column in under 5 minutes.

Fig. 3: HIC Analysis of H_2O_2 treated NIST mAb using a TSKgel Butyl-NPR Column



Instrument: Agilent 1100

Mobile phase: **A**: 2 mol/L ammonium sulfate + 100 mmol/L phosphate buffer, pH 7.0
B: 100 mmol/L phosphate buffer, pH 7.0

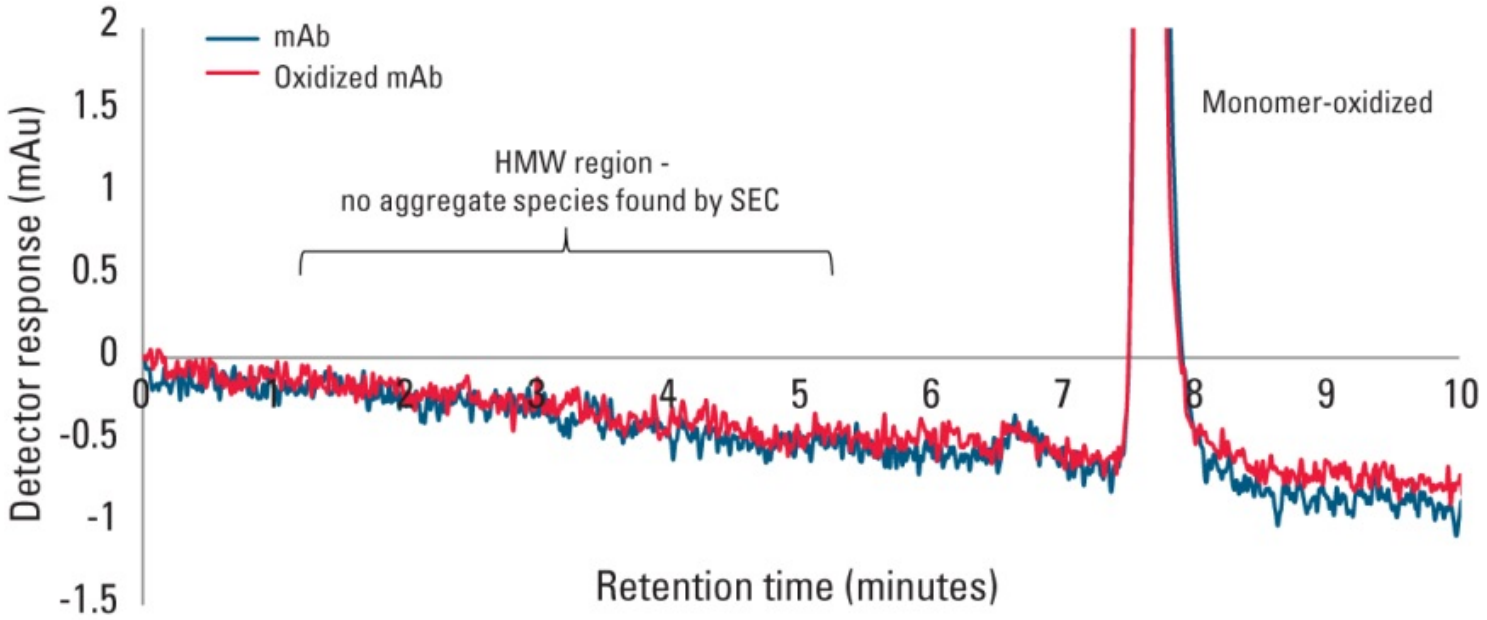
Gradient:

Time	%A	%B
0	100	0
15	0	100
20	0	100
20.1	100	0

Flow Rate: 0.35 mL/min
Detection: UV @ 280 nm
Injection vol.: 5 µL
Samples: NIST mAb (2 mg/mL)
NIST mAb (2 mg/mL) oxidized with 0.1% H_2O_2

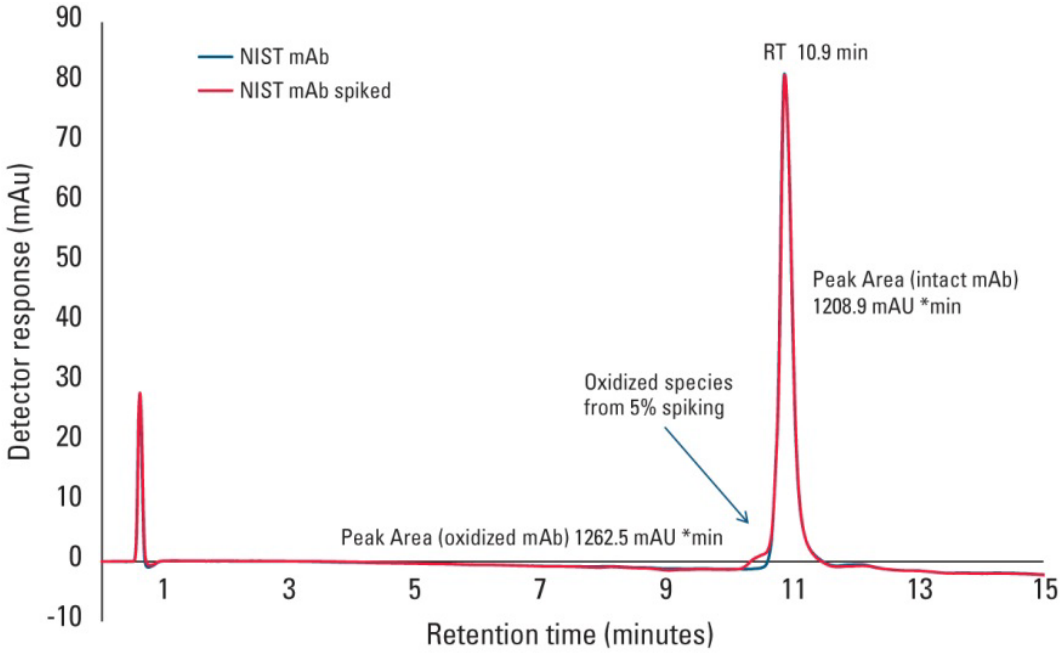
- Oxidation of NIST mAb with H_2O_2 results in conformational changes that impact the overall surface hydrophobicity of the molecule.
- The TSKgel Butyl-NPR column was able to elucidate these hydrophobic changes and showed clear variation in retention times associated with the H_2O_2 oxidized mAb compared to the non-oxidized species .

Fig. 4: Size Exclusion Chromatography (SEC) of Intact and Oxidized NIST mAb using a TSKgel UP-SW3000 Column



- In order to ensure the retention time shift is due to oxidation and not aggregation induced by the oxidizing reagent, SEC was performed. SEC is a historical technique widely employed for the detailed characterization of therapeutic proteins and can be considered as a reference and powerful technique for the qualitative and quantitative evaluation of aggregates.
- Two consecutive SEC analysis of NIST mAb oxidized by 0.1% H_2O_2 didn't yield any present aggregation. This result shows that the change in the overall chromatographic profile and the retention time is due to oxidation of mAb and not due to aggregation caused by the oxidizing reagent.

Fig. 5: HIC Analysis of Native and Oxidized NIST mAb using a TSKgel Butyl-NPR Column: Sensitivity



Instrument: Agilent 1100

Mobile phase: **A**: 2 mol/L ammonium sulfate + 100 mmol/L phosphate buffer, pH 7.0
B: 100 mmol/L phosphate buffer, pH 7.0

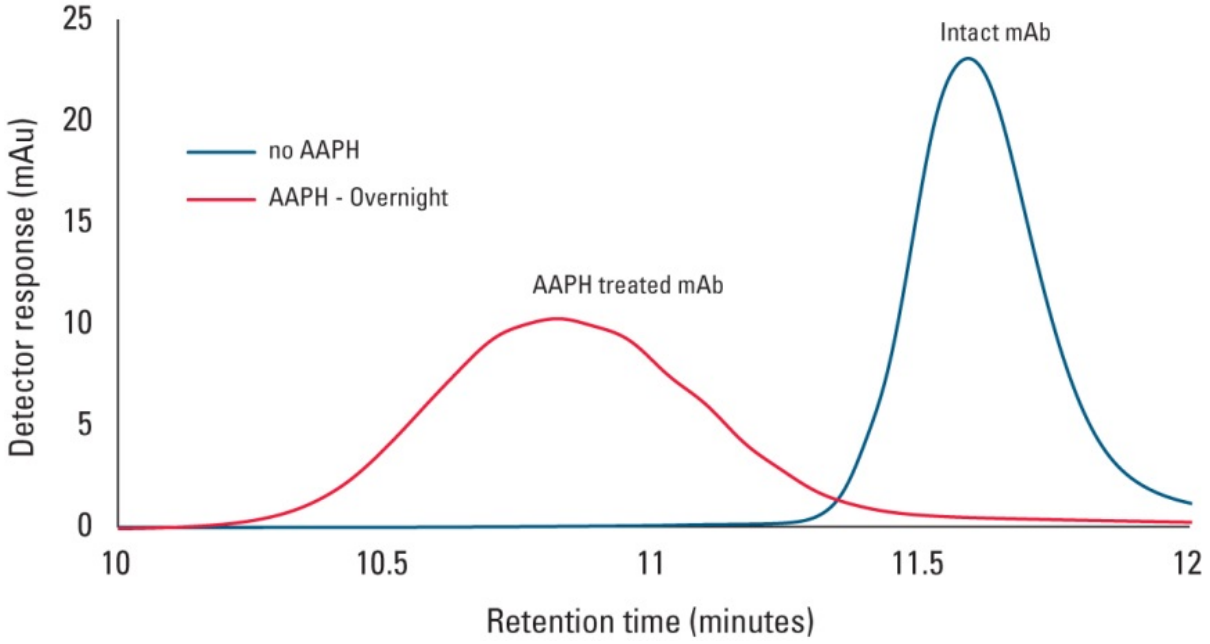
Gradient:

Time	%A	%B
0	100	0
15	0	100
20	0	100
20.1	100	0

Flow Rate: 0.35 mL/min
Detection: UV @ 280 nm
Injection vol.: 5 µL
Samples: NIST mAb (2 mg/mL)
NIST mAb (2 mg/mL) oxidized with 0.1% H_2O_2

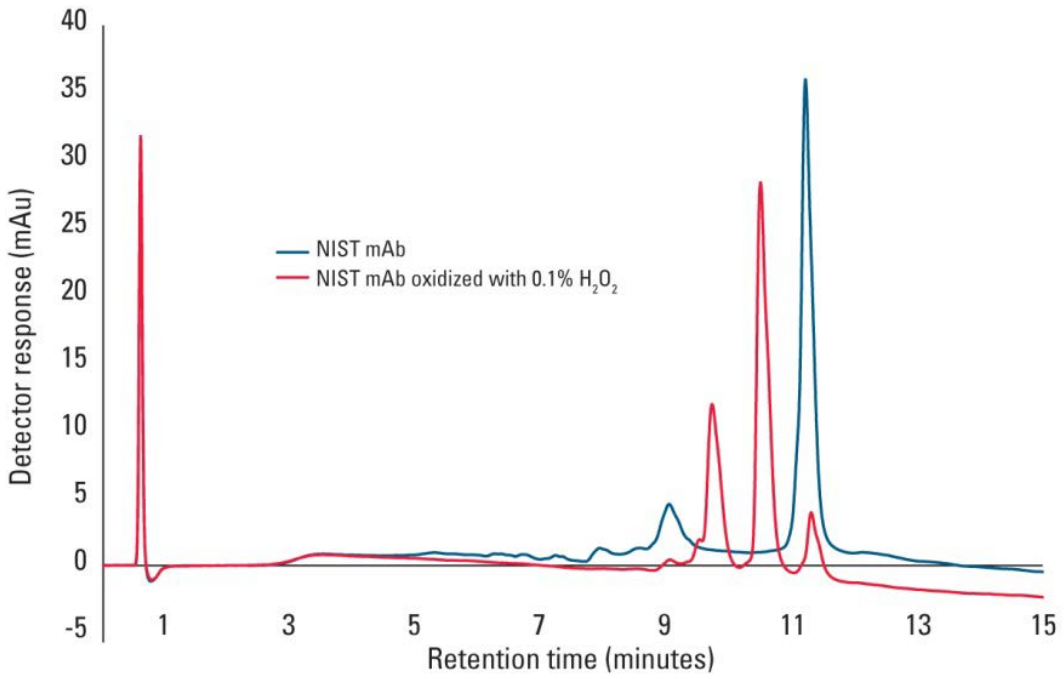
- In order to determine the utility of the TSKgel Butyl-NPR column in identifying low levels of oxidative species present in the sample, as opposed to identification of complete oxidation, non-oxidized NIST mAb was spiked with (5%) oxidized mAb.
- Peak area of oxidized mAb is ~5% (1263.4-1208.09)*100]/1208.9 = 4.57%), indicating that the TSKgel Butyl-NPR column can confidently identify low levels of oxidation present in the sample.

Fig. 6: HIC Analysis of Native and AAPH Treated NIST mAb using a TSKgel Butyl-NPR Column



- Oxidation of NIST mAb with AAPH results in conformational changes that impact the overall surface hydrophobicity of the molecule. The TSKgel Butyl-NPR column was able to elucidate these hydrophobic changes and showed clear variation in retention times associated with the AAPH oxidized mAb compared to the non-oxidized species .
- The chromatogram of AAPH oxidized NIST mAb sample yielded a broader peak when compared to H_2O_2 oxidation and exhibits a number of unresolved peaks likely due to oxidation of both the Met and Trp residues in this sample.

Fig. 7: Analysis of IdeS Reduced NIST mAb using a TSKgel Butyl-NPR Column



Instrument: Agilent 1100 with Chemstation

Mobile Phase: **A**: 2 mol/L ammonium sulfate + 100 mmol/L phosphate buffer, pH 7.0
B: 100 mmol/L phosphate buffer, pH 7.0

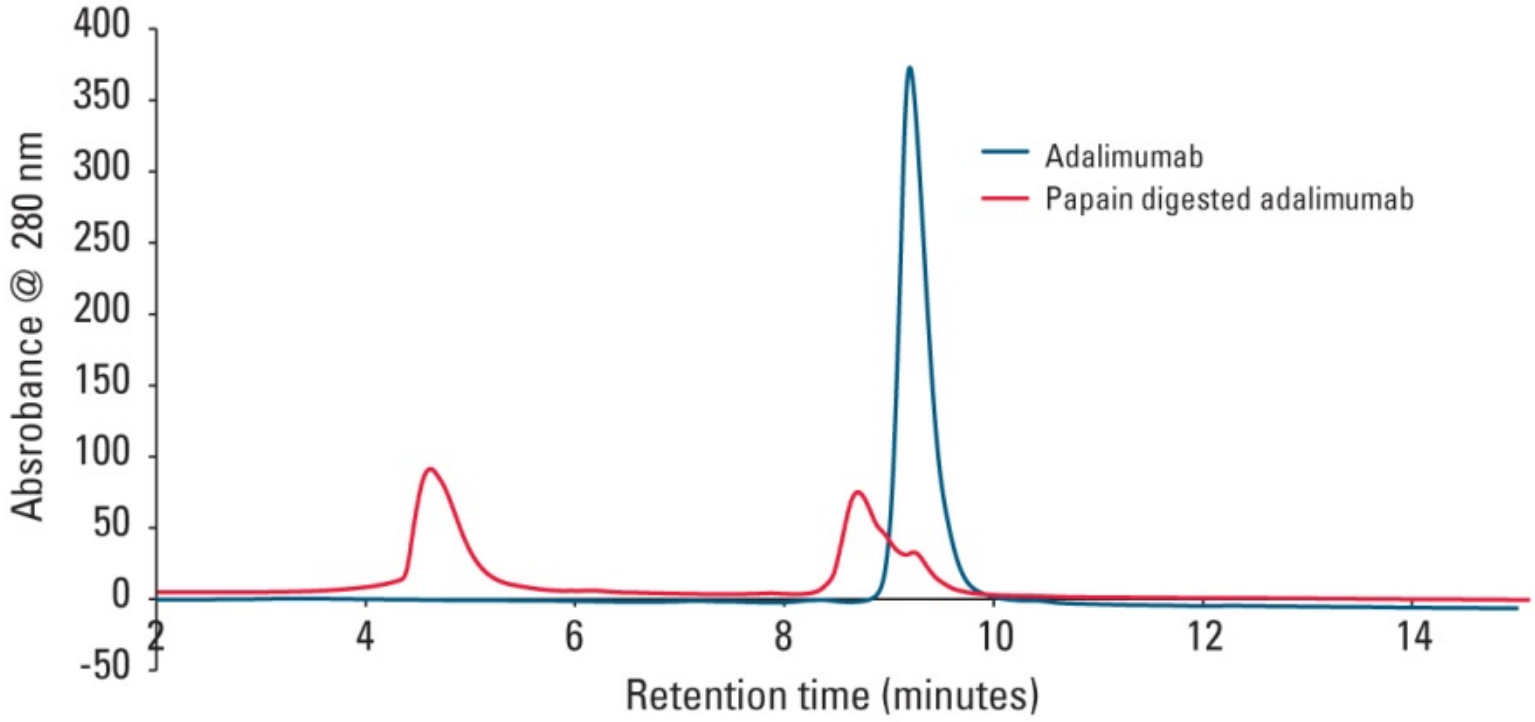
Gradient:

Time	%A	%B
0	100	0
15	0	100
20	0	100
20.1	100	0

Flow Rate: 0.35 mL/min
Detection: UV @ 280 nm
Injection vol.: 5 µL
Sample: NIST mAb (2 mg/mL)
NIST mAb (2 mg/mL) oxidized with 0.1% H_2O_2

- IdeS, a cysteine proteinase enzyme from Streptococcus pyo-genes, specifically cleaves human IgG in the hinge region between the two glycines of the constant sequence ELLGGPS (Ref:U. von Pawel-Rammingen, B.P. Johansson, L. Bjorck, EMBO J. 21 (2002) 1607) .
- TSKgel Butyl-NPR is able to resolve Fab and Fc fragments generated by IdeS digestion.

Fig. 8: Analysis of Papain Digested Adalimumab using a TSKgel Butyl-NPR HIC Column



- Fragmentation to the monovalent Fab fragment is carried out using papain digestion.
- The TSKgel Butyl-NPR column is able to resolve Fab and Fc fragments generated by papain digestion.

Conclusions

- The TSKgel Butyl-NPR column was able to identify a hydrophilic variant present in the native NIST mAb sample in under 5 minutes.
- The column shows a clear retention time shift for AAPH or H_2O_2 oxidized intact NIST mAb compared to the intact non-oxidized species, indicating utility in assessing the presence (or absence) of oxidation. Percent oxidation was able to be quantitated as low as 5% present oxidized species, with future experiments planned to determine the limit of quantitation.
- The results show that the TSKgel Butyl-NPR column can resolve Fab and Fc fragments generated by papain or IdeS digestion.

Future Work

- Pair forced oxidation with partial digestion to demonstrate the use of the TSKgel Butyl-NPR column to resolve oxidized and unoxidized mAb fragments.
- Use mass spectrometry to characterize the peaks in the HIC separation to better characterize the oxidation products; verify oxidation position by bottom up analysis of a tryptic digest.
- Explore parameters for method optimization and changes in selectivity on ether and phenyl HIC phases.