Characterization of Monoclonal Antibody Heterogeneity from Oxidation and Proteolysis by Analytical Hydrophobic Interaction Chromatography

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Introduction

- Oxidation of methionine or tryptophan residues has been associated with a decrease or elimination of biological activity of monoclonal antibody (mAb) biotherapeutics.
- Oxidation with hydrogen peroxide (H_2O_2) or 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) is accepted as a simulation of the chemical degradation/oxidative stress.
- Oxidation of the mAb results in conformational changes, which impacts surface hydrophobicity. Hydrophobic interaction chromatography (HIC) can be used to characterize the oxidized mAbs.
- HIC can also be used to resolve the products of partial digestion of mAbs, providing additional information.
- Here we report the use of a 4.6 mm ID × 3.5 cm, 2.5 µm, analytical HIC column for these purposes

Materials and Methods

Chemicals and Reagents

- Deionized (DI) water
- Isopropanol
- Potassium phosphate, monobasic, anhydrous($KH_2PO_4 \cdot H2O$)
- Sodium phosphate, dibasic, anhydrous (Na_2HPO_4)
- Ammonium sulfate $(NH_4)_2SO_4$
- Ethylenediaminetetraacetic acid (EDTA) tetra-sodium salt dihydrate ($C_{10}H_{14}N_2Na_2O_8$ $2H_2O$)
- L-cysteine
- Hydrogen peroxide (H_2O_2) 30%
- AAPH [2,2'-azobis(2-amidinopropane)]
- IdeS
- Papain, lyophilized

Materials and Methods

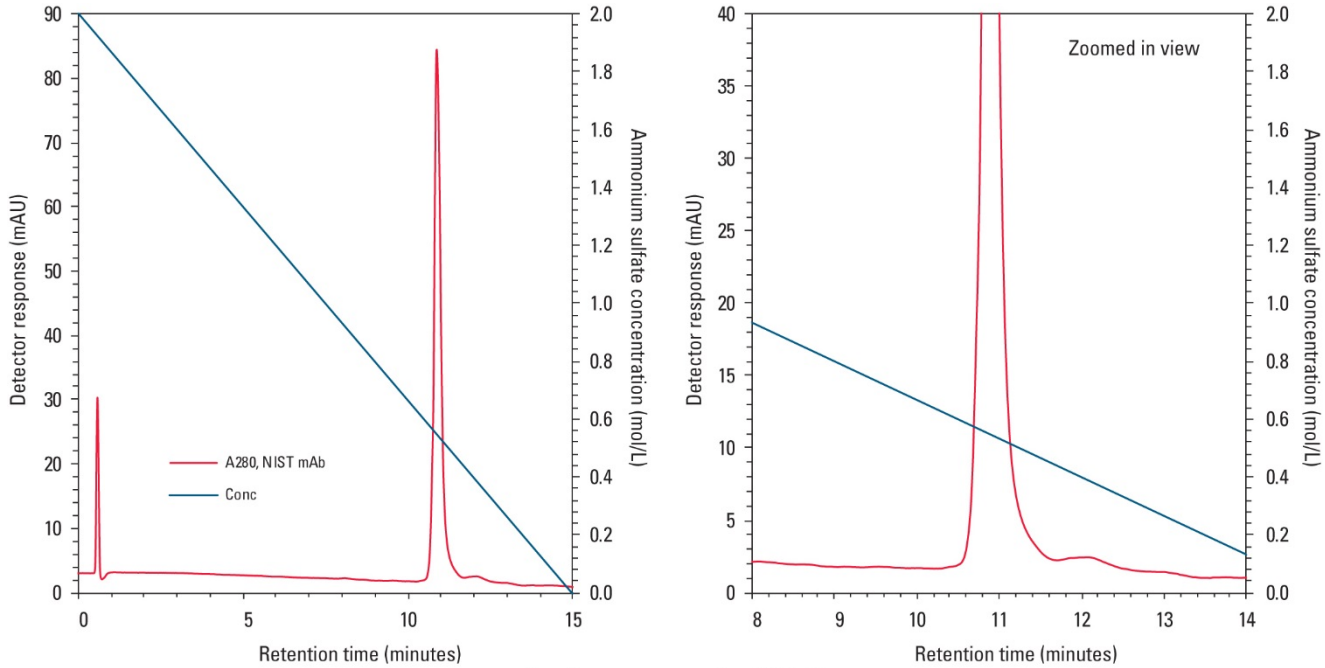
- **Preparation of 2× oxidation buffer:** 100 mL solution containing 360 mmol/L sodium chloride and 10 mmol/L sodium acetate was made in DI water. The pH was adjusted to 5.0 with 10 N HCl.
- **Oxidation of mAb with 0.1% H_2O_2 :** 20 µL of mAb solution was mixed with 50 µL of 2× oxidation buffer. H_2O_2 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.
- **Oxidation of mAb with AAPH:** 20 µL of mAb solution was mixed with 50 µL of 2× 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

Column:	TSKgel® Butyl-NPR, 2.5 µm (non-porous), 4.6 mm ID x 3.5 cm
Instrument:	Agilent 1100
Mobile phase A:	0.1 mol/L phosphate, 2.0 mol/L ammonium sulfate, pH 7.0
Mobile phase B:	0.1 mol/L phosphate, pH 7.0
Gradient:	as indicated
Flow rate:	as indicated
Temperature:	ambient
Detection:	UV @ 280 nm
Injection vol.:	as indicated
Sample:	mAb as indicated

HIC Separation of Oxidized mAb

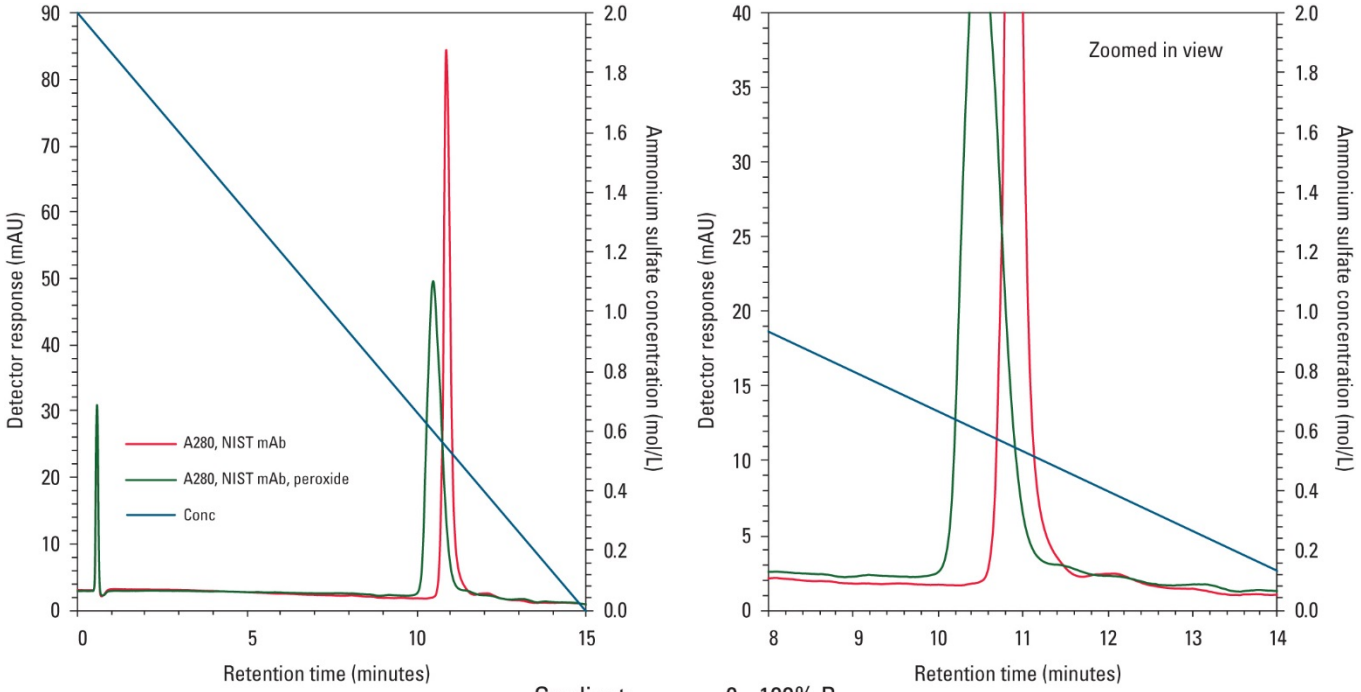
HIC Analysis of Intact NIST mAb using a TSKgel Butyl-NPR Column



Gradient: 0 - 100% B, 15 min
Flow rate: 0.35 mL/min
Injection vol.: 5 µL
Sample: NIST mAb, 2 mg/mL

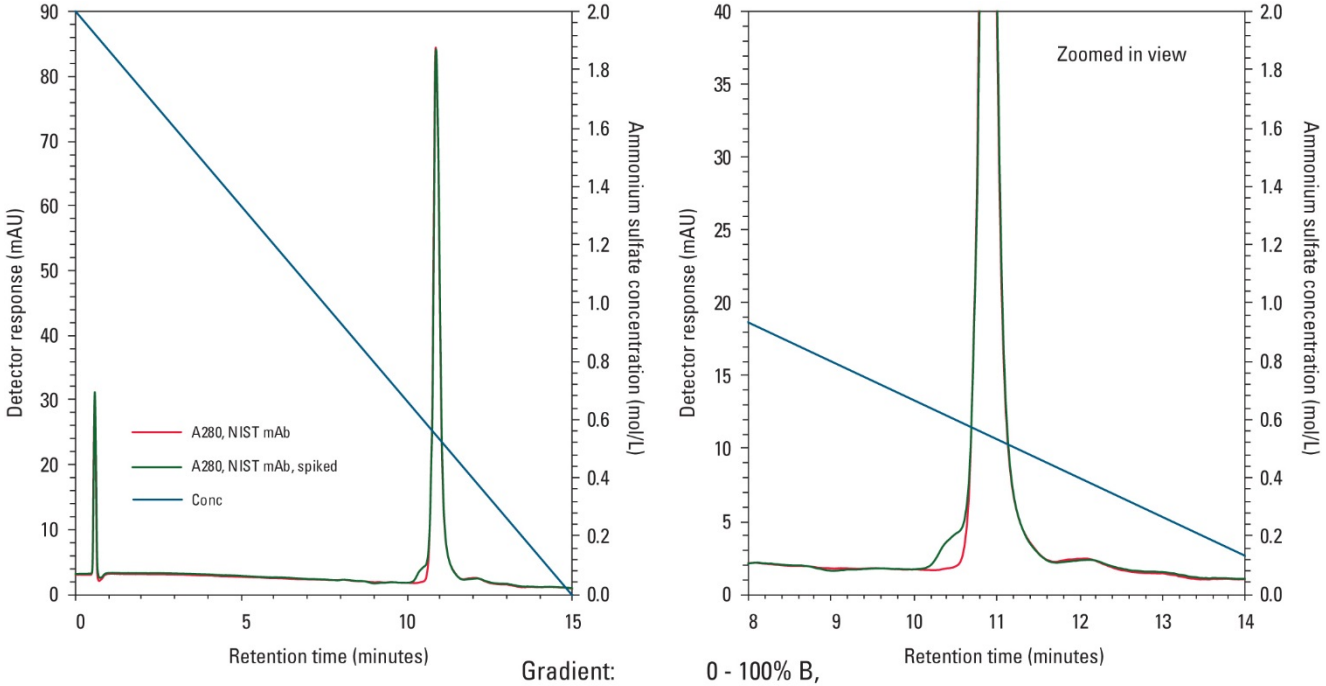
- Non-oxidized mAb is eluted from the TSKgel Butyl-NPR column by a reverse ammonium sulfate gradient.
- Elution occurs at T_R = 10.9 min, or approximately 530 mmol/L ammonium sulfate.

HIC Analysis of H_2O_2-Treated NIST mAb



- Oxidation of NIST mAb with H_2O_2 results in conformational changes that impact mAb surface hydrophobicity.
- Conditions could resolve H_2O_2-oxidized and non-oxidized species.

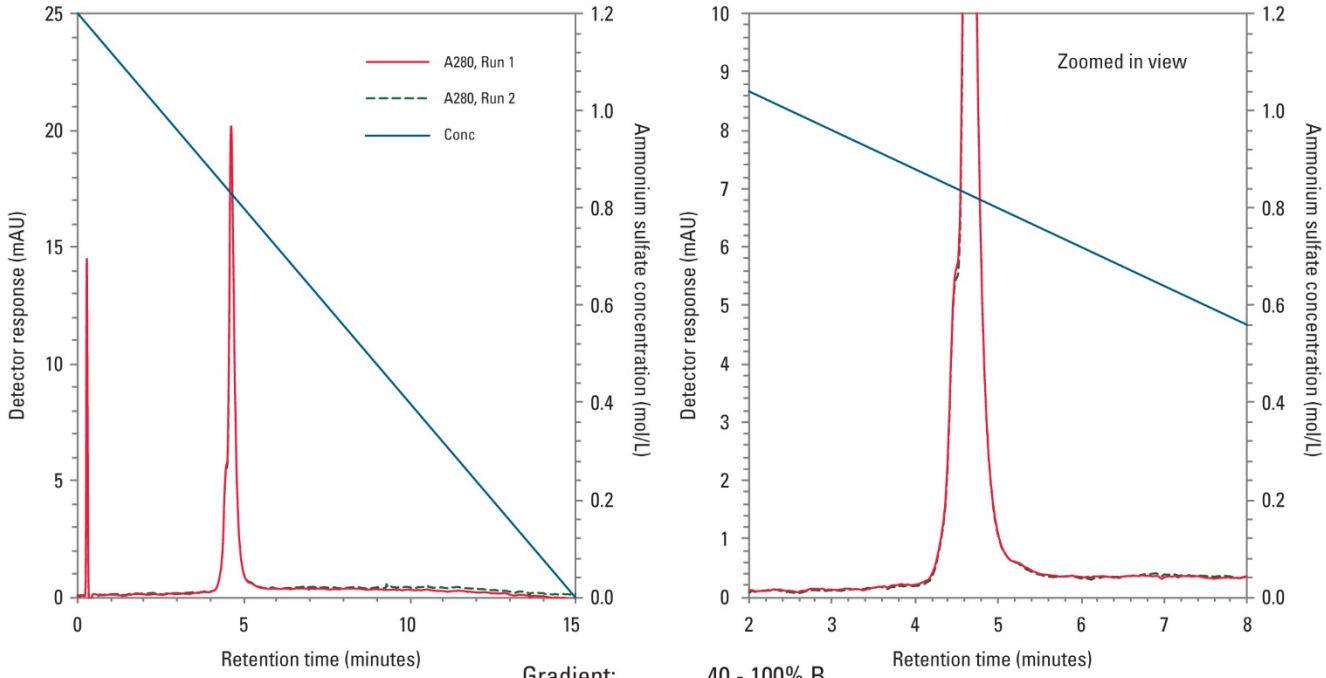
HIC Analysis of Native and Oxidized NIST mAb – Sensitivity



Gradient:	0 - 100% B, 15 min
Flow rate:	0.35 mL/min
Injection vol.:	5 µL
Sample:	NIST mAb, 2 mg/mL

- Non-oxidized NIST mAb was spiked with 5% peroxide-oxidized mAb.
- Peak area of oxidized mAb is ~5% (1263.4-1208.09)*100]/1208.9 = 4.57%)
- Column can identify low levels of oxidation present in the sample.

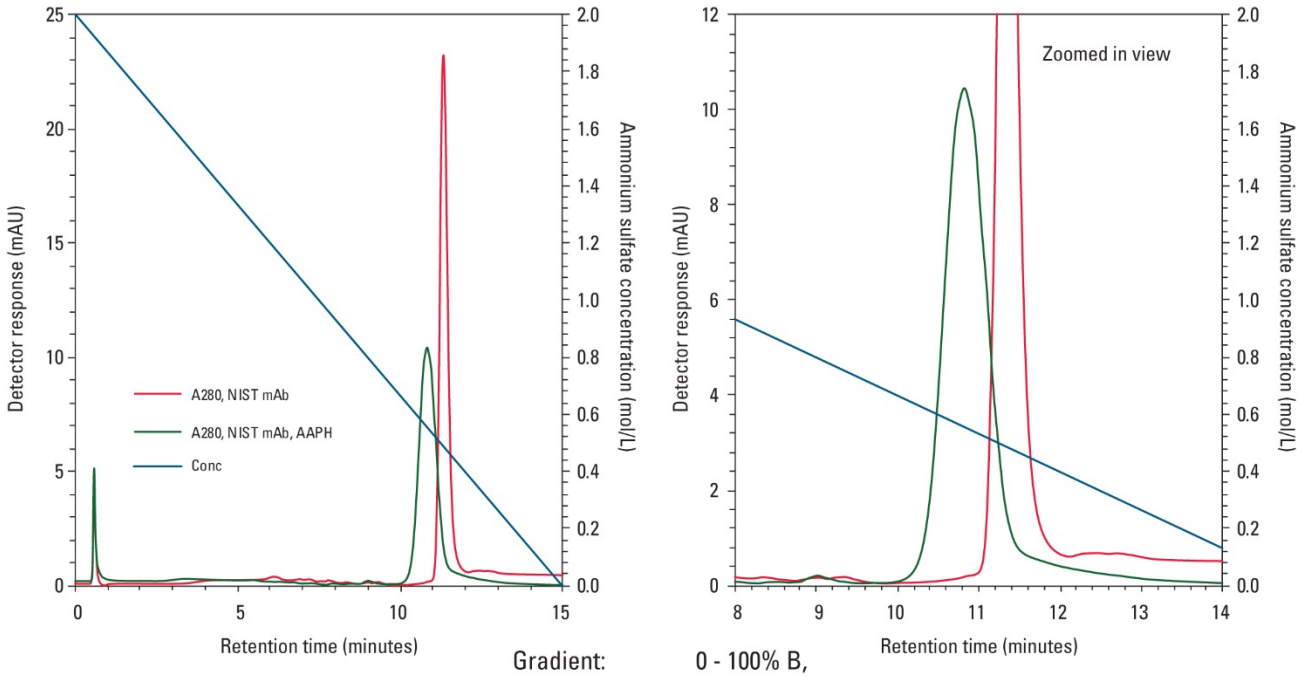
HIC Analysis of NIST mAb Oxidized under Ambient Conditions – Optimized Gradient



Gradient:	40 - 100% B, 15 min
Flow rate:	0.35 mL/min
Injection vol.:	5 µL
Sample:	NIST mAb, 2 mg/mL

- Hydrophobic variant of NIST mAb generated by long-term exposure to ambient temperatures.
- Conditions optimized for better resolution and faster elution.

HIC Analysis of Native and AAPH-Treated NIST mAb



- Oxidation of NIST mAb with AAPH impacts surface hydrophobicity.
- Conditions could resolve AAPH-oxidized and non-oxidized species.
- AAPH-oxidized NIST mAb sample yielded broader peak vs. H_2O_2 oxidation.
- Shows a number of unresolved peaks -- oxidation of both Met and Trp residues

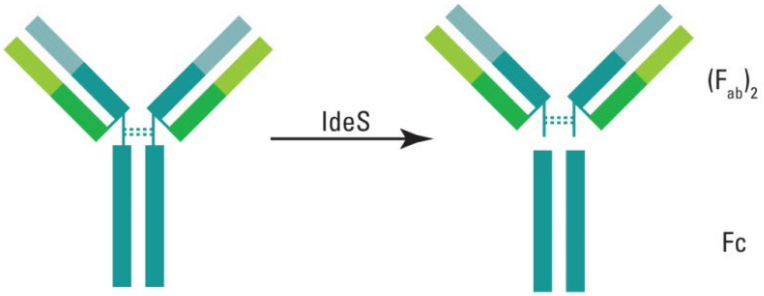
Size Exclusion Chromatography (SEC) of Intact and Oxidized NIST mAb using a TSKgel UP-SW3000 Column

- 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 did not 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.

HIC Separation of mAb Fragments

Mechanism of Papain and IdeS Fragmentation

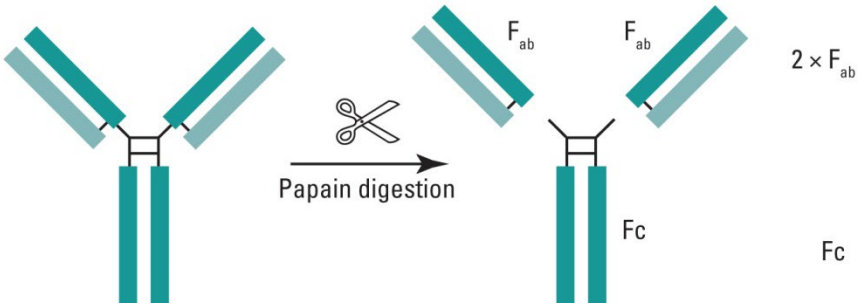
Schematic diagram of fragmentation of a mAb by IdeS
(Ref: mAbs 2014, 6:4, 879–893)



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

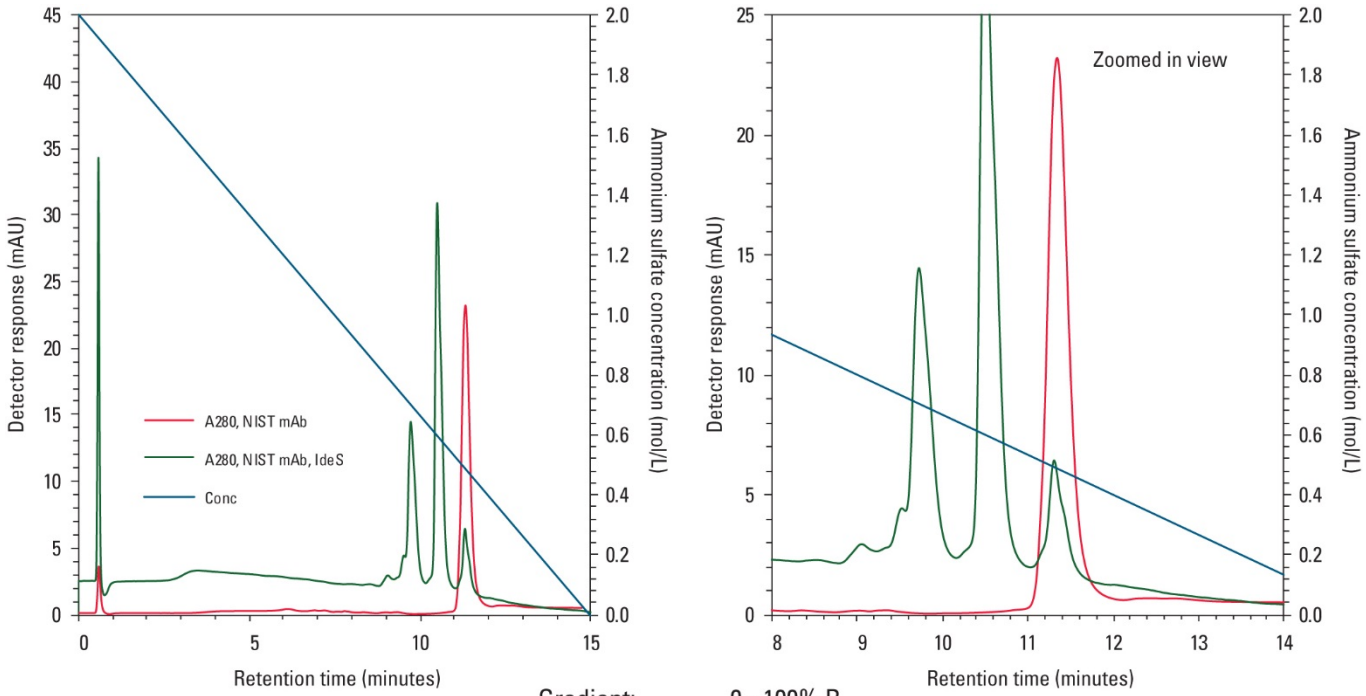
Schematic diagram of fragmentation of a mAb by papain



Materials and Methods

- **Preparation of 2× 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 with IdeS:** 20 µL of mAb solution was mixed with 50 µL of 2× 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 with papain:** 2 mg/mL papain solution was prepared in DI water. 20 µL of mAb was mixed with 50 µL of 2× 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.

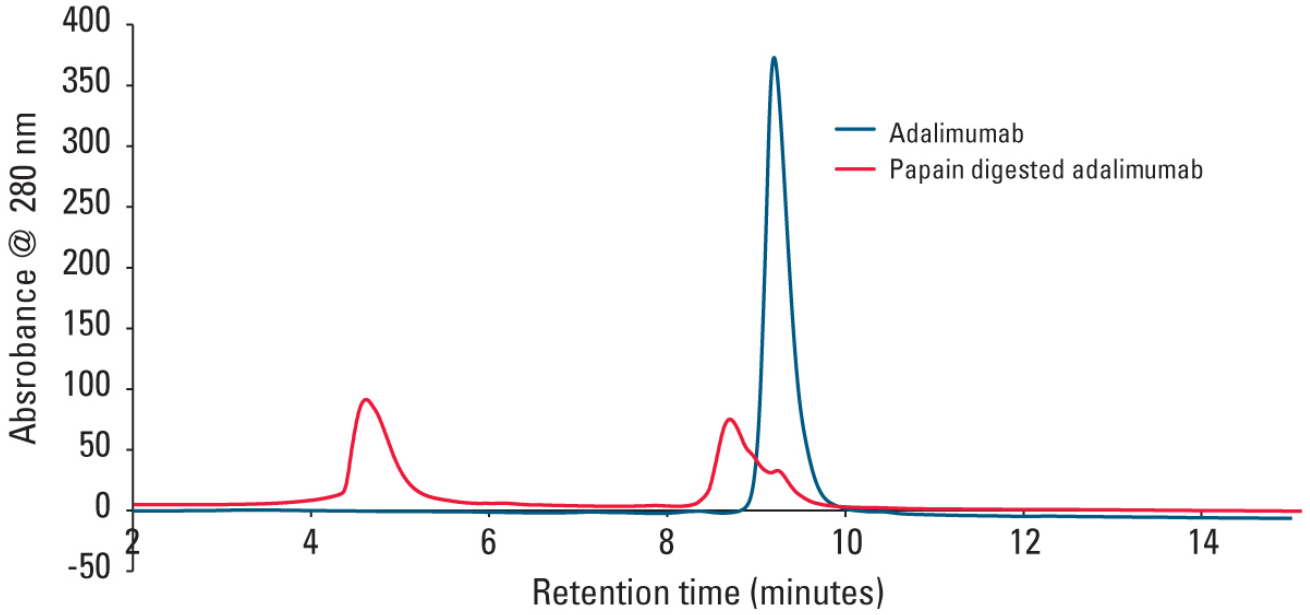
Analysis of IdeS Reduced NIST mAb using a TSKgel Butyl-NPR Column



Gradient: 0 - 100% B, 15 min
Flow rate: 0.35 mL/min
Injection vol.: 5 µL
Sample: NIST mAb, 2 mg/mL

- IdeS cleaves human IgG in the hinge region between the two glycines at sequence ELLGGPS (U. von Pawel-Rammingen, B.P. Johansson, L. Bjorck, EMBO J. 21 (2002) 1607)
- Conditions resolve Fab and Fc fragments generated

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

- A clear retention time shift is noticed for AAPH- or H_2O_2-oxidized NIST mAb vs. intact, non-oxidized species. This indicates the column's utility in assessing the presence or absence of oxidation.
- Percent oxidation was able to be quantitated as low as 5% -- future experiments planned to determine the limit of quantitation.
- Column can resolve Fab and Fc fragments generated by papain treatment or $(Fab)_2$ and Fc fragments generated by IdeS treatment.

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

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