Application Note

Biologics Research

AdvanceBio HIC: a Hydrophobic HPLC Column for Monoclonal Antibody (mAb) Variant Analysis

Using the Agilent 1260 Infinity II Bio-Inert LC

Authors

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Abstract

This Application Note describes the separation of oxidized monoclonal antibody (mAb) variants from their native form using the Agilent AdvanceBio HIC column. Oxidation of exposed amino acid side chain residues such as methionine, cysteine, and tryptophan is a common degradation pathway for monoclonal antibodies, and presents a major analytical challenge in biotechnology. Often, oxidized mAbs have decreased potency compared to their native form[1]. Therefore, to ensure the therapeutic efficacy of the mAb products, analysis of such degradation is critical. Oxidation of amino acid residues on an mAb can alter the hydrophobic nature of the mAb by the increase in polarity of the oxidized form, or also due to resulting conformational changes[2]. HPLC methods for separating biomolecules based on differences in hydrophobicity include reversed-phase and hydrophobic interaction chromatography (HIC). HIC can be applied to characterize mAb variants resulting from post-translational modifications (PTMs). The AdvanceBio HIC column provides excellent resolution of oxidized mAb variants from unmodified forms, and can resolve oxidized species without mAb digestion into subunits or other sample preparation methods.

Introduction

mAbs and related products such as antibody drug conjugates (ADCs) and bispecific antibodies (bsAbs) are the fastest growing classes of biotherapeutics. Recombinant mAbs are subject to many PTMs during processing, delivery, and storage. Among these modifications, oxidation of exposed amino acid side chains such as methionine (Met) and tryptophan (Trp) is a common occurrence. Various researchers have reported that oxidation of mAbs has an adverse effect on product shelf life and bio-activity[1,2]. Therefore, developing analytical methods to detect oxidized mAb variants has gained interest. The sulfoxide and sulfone side chains of methionine-oxidized mAb products are larger and more polar compared to the native form, which may alter protein structure, stability, and biological function. Hydrophobicity-based HPLC methods, such as reversed-phase liquid chromatography (RPLC) and HIC, are often used to characterize mAb variants. Recently, several studies have indicated that HIC can be applied to monitor oxidation of recombinant mAbs with reasonable selectivity and ease, as an excellent alternative to RPLC[3].

HIC is similar to RPLC in that separation of analytes is based on hydrophobic interactions with the stationary phase. The elution order in HIC enables proteins to be ranked based on their relative hydrophobicity. Unlike RPLC, HIC employs nondenaturing conditions, does not require the use of organic solvents or high temperatures, and separations are carried out at physiological pH, allowing for the preservation of protein structure. Thus, conformational changes in the native form of the protein may be analyzed using HIC[4].

AdvanceBio HIC is a silica-based HPLC column designed for the separation of mAbs and related products. Its unique proprietary bonded phase chemistry provides high resolution and desired selectivity for the analysis of mAbs and mAb variants. This Application Note describes the separation of oxidized NIST mAb variants using an AdvanceBio HIC column.

Experimental

Equipment and materials

All chemicals and reagents were HPLC grade or higher, and were obtained from Sigma-Aldrich (now Merck) or VWR Scientific. Humanized IgG1k mAb sample (product item no. 8671) was obtained from NIST SRM Standards. Water was purified using a Milli-Q A10 water purification system (Millipore).

Instrumentation

Agilent 1260 Infinity II bio-inert LC comprising:

- Agilent 1260 Infinity II bio-inert pump (G5654A)
- Agilent 1260 Infinity II bio-inert multisampler (G5668A) with sample cooler (option no. 100)
- Agilent 1260 Infinity II multicolumn thermostat (G7116A) with bio-inert heat exchanger (option no. 019)
- Agilent 1260 Infinity II diode array detector WR (G7115A) with bio-inert flow cell (option no. 028)

Software

Agilent OpenLab 2.2 CDS

mAb Oxidation with *t*-BHP treatment

A solution of 1 mL of NIST mAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 70 % *tert*-butyl hydroperoxide (*t*-BHP) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Reaction conditions used to obtain Figure 6 data: 2 % (v/v) of 70 % *t*-BHP solution was added to a 1-mL sample of NIST mAb (1 mg/mL), and the reaction mixture was injected onto the column. The sample vial was held at 7 °C, and multiple injections from the same vial were carried out.

mAb Oxidation with H_2O_2 treatment

A solution of 1 mL of NIST mAb (1 mg/mL) in formulation buffer was incubated with 0.2 % (v/v) of 50 % hydrogen peroxide (H_2O_2) solution at room temperature for 24 hours. Residual oxidant was removed using Amicon Ultra-10 centrifugal filters with a molecular weight cut-off of 10 kDa. Samples were then buffer exchanged with 50 % mobile phase B.

Results and discussion

Protein oxidation is frequently monitored in stability studies or stressed samples during formulation development (for example, samples exposed to a chemical oxidant such as hydrogen peroxide (H_2O_2), UV light, or metal ions). In this study, *t*-BHP and H_2O_2 were used as chemical oxidants to promote oxidation of NIST mAb samples. It was previously reported that both of these reagents tend to specifically oxidize Met side chain residues of the mAb. H_2O_2 more readily oxidizes less accessible, buried residues, whereas *t*-BHP is known to target more surface-exposed Met residues[7]. Figure 1 illustrates the reaction scheme for Met oxidation induced by chemical oxidants.

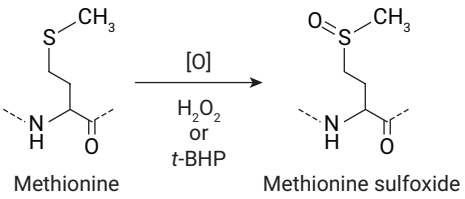

Figure 1. Methionine oxidation induced by chemical oxidant.

The NIST mAb (humanized IgG1k) amino acid sequence in Figure 2 shows that there are six possible surface-accessible Met residues located on both heavy chains of the mAb. Based on prior studies for most human IgG1-subclass antibodies, Met residues localized to the CH_2 and CH_3 domains of the antigen binding, or Fc, region are known to be highly susceptible to oxidation[5]. In the case of NIST mAb, Met 255 and Met 431 correspond to the amino acid residues prone to oxidation. This is depicted by the illustration in Figure 3.

Method conditions

	HPLC conditions
Column	AdvanceBio HIC, 4.6 × 100 mm (p/n 685975-908)
Mobile phase	Eluent A) 50 mM sodium phosphate, pH 7.0 Eluent B) 2 M ammonium sulfate in 50 mM sodium phosphate, pH 7.0
Flow rate	0.3 to 0.5 mL/min
Column temperature	25 °C
Injection volume	5 µL
Final sample concentration	1 mg/mL
Detection	UV, 220 nm
Gradient profile	Flow rate: 0.5 mL/min Time %A %B 0 50 50 20 100 0 25 100 0 30 50 50 40 50 50
Shallower gradient	Flow rate: 0.3 mL/min Time %A %B 0 40 60 40 90 10 45 90 10 50 40 60 60 40 60

Heavy chain

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QVTLRESGPA LVKPTQTLTL TCTFSGFSLS TAGMSVGWIR QPPGKALEWL ADIWWDDKKH YNPSLKDRLT
ISKDTSKNQV VLKVTNMDPA DTATYYCARD MIFNFYFDVW GQGTTVTVSS ASTKGPSVFP LAPSSKSTSG
GTAALGCLVK DYFPEPVTVS WNSGALTSGV HTFPAVLQSS GLYSLSSVVT VPSSSLGTQT YICNVNHKPS
NTKVDKRVEP KSCDKTHTCP PCPAPELLGG PSVFLFPPKP KDTLMISRTP EVTCVVVDVS HEDPEVKFNW
YVDGVEVHNA KTKPREEQYN STYRVVSVLT VLHQDWLNGK EYKCKVSNKA LPAPIEKTIS KAKGQPREPQ
VYTLPPSREE MTKNQVSLTC LVKGFYPSDI AVEWESNGQP ENNYKTTPPV LDSDGSFFLY SKLTVDKSRW
QQGNVFSCSV MHEALHNHYT QKSLSLSPGK
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Light chain

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DIQMTQSPST LSASVGDRVT ITCSASSRVG YMHWYQQKPG KAPKLLIYDT SKLASGVPSR FSGSGSGTEF
TLTISSLQPD DFATYYCFQG SGYPFTFGGG TKVEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP
REAKVQWKVD NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL SSPVTKSFNR GEC
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Figure 2. NIST mAb amino acid sequence.

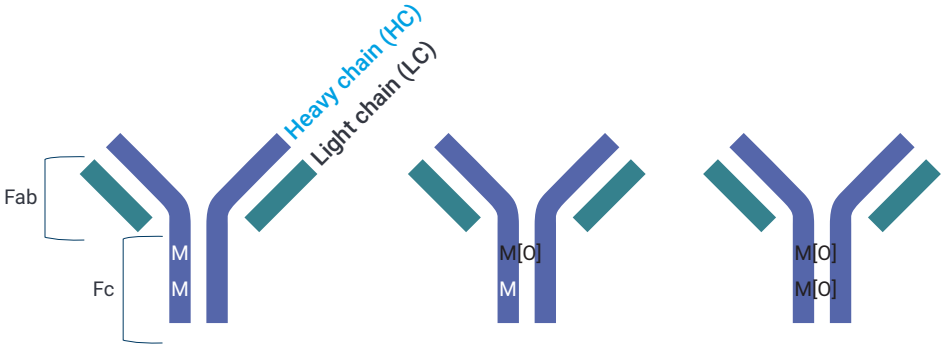
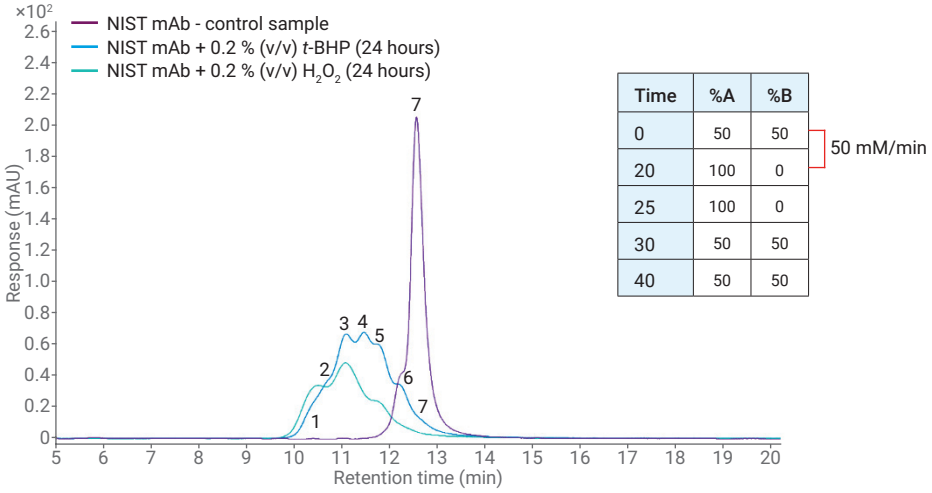


Figure 3. Methionine residues located in Fc region are most susceptible to oxidation in human IgG1 mAbs.

An AdvanceBio HIC column was able to differentiate oxidized mAb variants from the untreated mAb sample under low salt starting conditions. Oxidation of the NIST mAb with *t*-BHP under reported experimental conditions resulted in multiple peaks with shorter retention times, presumably due to conformational change. The HIC chromatogram (Figure 4) showing earlier retained peaks labeled 1 to 6 likely indicates the result of oxidized Met residues on the mAb, and peak 7 with a retention time of approximately 12.6 minutes, corresponds to nonoxidized mAb. For the H_2O_2-treated mAb sample, complete oxidation occurred, with three peaks eluting in a shorter retention time, indicating more aggressive oxidation of Met residues. These differences in the chromatograms of the IgG1k mAb sample incubated with two different oxidation reagents suggest that reactivity is governed by solvent accessibility of the Met residues and steric limitations of the oxidizing agent, as previously reported[6].

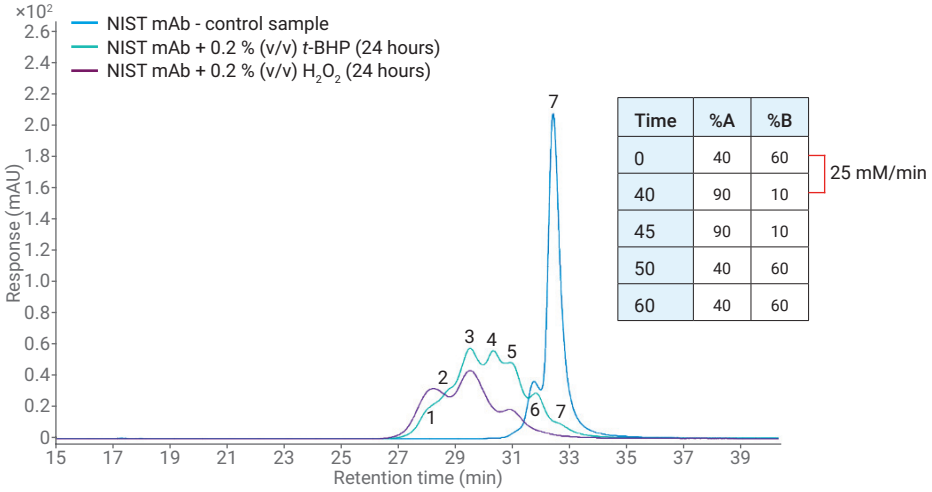
To further improve the resolution, a slower and shallower gradient was used. Using a flow rate of 0.3 mL/min and a starting ammonium sulfate concentration of 1.2 M with a lower gradient rate of 25 mM/min, better resolution was achieved with a relatively short analysis time (Figure 5). In this chromatogram, multiple mAb-oxidized species are clearly observed from the untreated mAb sample.



Time	%A	%B
0	50	50
20	100	0
25	100	0
30	50	50
40	50	50

50 mM/min

Figure 4. Separation of oxidized NIST mAb variants using lower starting salt concentration.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

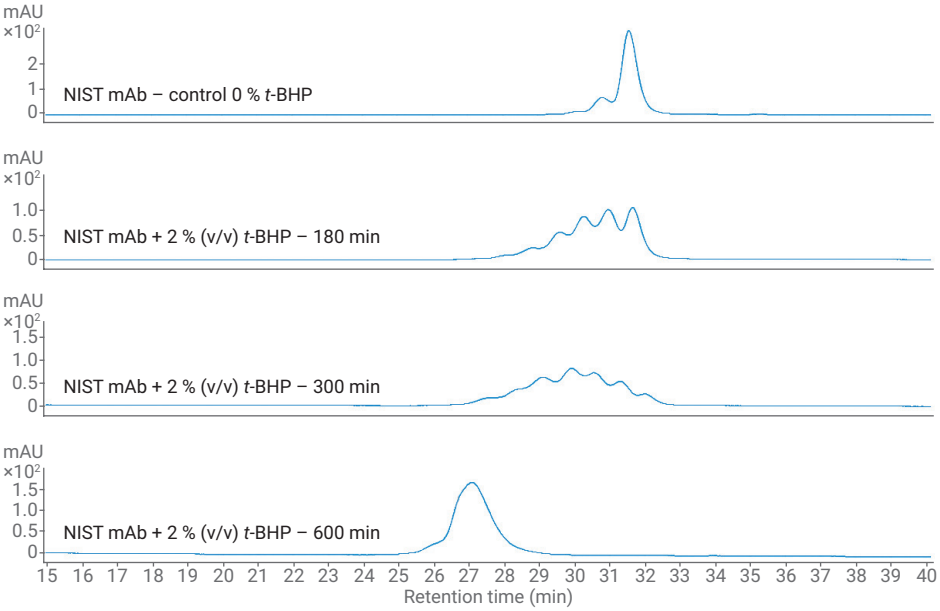
25 mM/min

Figure 5. Separation of oxidized NIST mAb variants using a shallow gradient.

In Figure 6, the NIST mAb sample was incubated with 2 % (v/v) *t*-BHP, and the oxidation reaction was monitored at various time points using shallower gradient conditions. As represented by an overlay of chromatograms, the mAb oxidation progressed with *t*-BHP incubation time. Multiple mAb oxidation species were observed within a few hours of the oxidation reaction. This suggested that surface-accessible Met residues in both heavy chains of the mAb sample might be oxidized randomly, which was previously reported[5]. Further oxidation of the mAb sample after 10 hours of reaction led to a broad peak, indicating forced oxidation. It has previously been speculated that oxidation of deeply buried Met residues can lead to a more dramatic structural change, which may cause the mAb to partially unfold[7]. Partially unfolded mAb is likely to have more conformational variation, resulting in a broader peak with a large retention time shift.

Conclusions

The AdvanceBio HIC column demonstrated the separation of oxidized mAb variants from its native form. Using the AdvanceBio HIC column, optimal separation of oxidized mAb variants can be achieved using slower flow rates and shallower gradient conditions, while maintaining relatively short analysis times.



Time	%A	%B
0	40	60
40	90	10
45	90	10
50	40	60
60	40	60

25 mM/min

Figure 6. Monitoring the *t*-BHP oxidized mAb reaction.

References

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