Journal of Chromatography A, 1431 (2016) 154–165

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

Analysis of monoclonal antibody oxidation by simple mixed mode chromatography

Jorge Alexander Pavon, Xiaojuan Li, Steven Chico, Umesh Kishnani, Soundara Soundararajan, Jason Cheung, Huijuan Li, Daisy Richardson, Mohammed Shameem, Xiaoyu Yang*

Bioprocess Development, Merck Research Laboratories, Merck & Co., Inc., Kenilworth, NJ 07033, USA

ARTICLE INFO

Article history:
Received 4 September 2015
Received in revised form
21 December 2015
Accepted 26 December 2015
Available online 13 January 2016

Keywords:
Mixed mode chromatography
Methionine
Tryptophan
Oxidation
Monoclonal antibody

ABSTRACT

Analysis of oxidation of monoclonal antibodies (mAbs) in most cases relies on peptide mapping and LC–MS, which is time consuming and labor-intensive. A robust chromatography based method that is able to resolve and quantitate mAb oxidation variants due to oxidized methionine or tryptophan is highly desired. Here we developed a novel mixed mode chromatography method using the unique property of Sepax Zenix SEC-300MK column to analyze mAb oxidation levels. The separation of oxidized species relied upon the mixed mode of size exclusion and hydrophobic interaction between the resin and antibodies. The chromatography was performed in a regular SEC mobile phase, PBS, containing NaCl at a concentration (0–2.4 M) specific for individual antibodies. This method was able to resolve and quantitate the oxidized antibodies as prepeaks, of either methionine-oxidized species induced by the common oxidants TBHP, tryptophan-oxidized species triggered by AAPH, or oxidized species by UV photo-irradiation. The prepeaks were further characterized by SEC-MALLS as monomers and confirmed by LC–MS as oxidized antibody variants with a mass increase of 16 or 32 Da. This method has been successfully applied to monitor multiple monoclonal antibodies of IgG1, IgG2, and IgG4 subclasses.

1. Introduction

Monoclonal antibodies (mAbs) are the most successful biologic drugs developed for treating a wide variety of diseases including cancers, arthritis, immunological disorders; their success is based on their overall stability and manufacturability [1–5]. Although mAbs are a very stable class of therapeutic proteins, and normally exhibit very good pharmacokinetics profiles in patients, they are still susceptible to a variety of post-translational modifications (PTMs) during production and storage. The observed PTMs include glycation, N-terminal glutamine cyclization and C-terminus lysine cleavage, deamidation, isomerization, and oxidation [6,7]. Among these modifications, oxidation is commonly detected and results from reaction of reactive oxygen species with solvent-exposed amino acid residues including methionine, tyrosine, tryptophan, and cysteine residues [6]. The mechanisms involved in the oxidation process are likely complex and case specific. Oxidation can induce the structural changes that could potentially impact biological efficacy, clearance, safety, and immunogenicity of mAbs. Oxidation, specifically of methionine and tryptophan side chains, has been shown to affect antibody binding to Fc receptors [8,9] and antigens [10], and to impact mAb stability and half-life [11]. Therefore, it is critical to monitor and characterize oxidation during different stages of drug development and production [7].

Monitoring the levels of oxidized species relies almost exclusively on liquid chromatography–mass spectrometry (LC–MS)-based methodologies, which are essential for characterizing biologics because of the sensitivity, resolution, selectivity, and specificity of the technique [12–14]. Specifically, peptide mapping via reverse phase liquid chromatography coupled with mass spectrometry is a very effective method for monitoring oxidation and other PTMs and identifying the modification sites. However, it has some limitations as the sample preparation process for RP-LC–MS is lengthy, and in some cases the chromatographic conditions such as high temperature, organic solvents, and acidic pH could induce oxidation artifacts. Although hydrophobic interaction chromatography (HIC) and Protein A chromatography have been used for analysis of antibody oxidation in some cases [15–17], these methods require long chromatographic run times, and have the limited power for separating oxidized protein variants.

* Corresponding author at: Bioprocess Development, Merck Research Laboratories, Merck & Co., Inc. 2000, Galloping Hill Road, Kenilworth, NJ 07033, USA.
E-mail address: xiaoyu.yang@merck.com (X. Yang).

http://dx.doi.org/10.1016/j.chroma.2015.12.068
0021-9673/

Recently, mixed mode SEC chromatography has been used for analysis of oxidized tryptophan variants of mAbs using Waters BEH 200 [18] and IgG4 half molecule exchange using Sepax Zenix SEC-300 [19,20]. In this paper, we present the development of a quick and efficient mixed mode liquid chromatography for general quantitative analysis of mAb oxidation including methionine and tryptophan-oxidized antibody variants. The method utilizes a Sepax Zenix SEC-300MK column that is able to separate oxidized variants from native antibody based on size and hydrophobicity, and can be readily implemented in routine analysis for monitoring mAb oxidation during stability and forced degradation studies.

2. Materials and methods

2.1. Materials

Tert-butyl hydroperoxide (TBHP), 70% solution (PN#180340050) was purchased from Acros Organics, and 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) (PN#44914), phosphate buffered saline (PBS) (8.1 mM Na_2HPO_4 and 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH 7.4) were from Sigma–Aldrich. Amicon Ultra filter (10,000 MW cutoff, #UFC901096) was from Millipore. Zeba Spin columns 2 mL (PN#89891) and 5 mL (PN#89893) were purchased from Fisher Scientific. SRT-SEC-300 (7.8 × 300 nm, PN#215300-7830) and Zenix SEC-300 MK (7.8 × 300 nm, PN# 213300-7830MK) were from Sepax Technology. YMC-Pack Diol-200 column (8.0 mm × 300 mm, PN# DS20S05-3008WT) was from YMC.

2.2. Monoclonal antibodies (IgGs)

Antibodies [mAbs A (IgG1), B (IgG1), C (IgG4), D (IgG1) and E (IgG2)] were produced in CHO cells, and purified via a multiple-column purification process including Protein A chromatography. Antibodies were kept in their respective buffers for stability. The protein concentrations were determined by UV absorbance at 280 nm, and most mAbs were provided at 25 mg/mL.

2.3. Stressing of mAbs to generate oxidized species

2.3.1. Light stress

Each IgG under investigation (200 μL per exposure condition) was aliquoted into quartz cuvettes or HPLC glass vials. Light exposure was conducted in a photostability chamber (Caron model 6545-2) at 25 °C. The levels selected ranged from 0X to 2X light exposure, where 1X = light exposure level equivalent to 1.2 million lux h of white light and 200 W h/m^2 of UV light based on ICH guidelines. After each designated exposure level was reached, the samples were covered and placed inside a dark box.

2.3.2. TBHP treatment

The antibodies (mAbs A, B, and C) were diluted to 5.0 mg/mL and incubated at 25 °C for various times in an HPLC vial in the presence of 0.1% TBHP, and then injected for analysis. For isolation of oxidized prepeaks, the antibody samples were buffer exchanged into their respective formulation buffer with a 10 mL Zeba spin desalting column (7 kDa MWCO). The column was first equilibrated with formulation buffer three times, and the entire sample (4 mL or 6 mL) was loaded into the column and centrifuged for four minutes at 1000 × *g*.

2.3.3. Forced oxidation with AAPH

For oxidation of mAb B and mAb E, antibodies at 5.0 mg/mL were treated with 2 mM of the 2,2′-azobis(2-methylpropionamidine) dihydrochloride (AAPH) in a HPLC vial at 40 °C and injected into the HPLC for analysis at different time points. Control samples of mAb B and mAb E (5.0 mg/mL) without AAPH were incubated in the sample compartment at 40 °C and injected at time zero (T0) and after 10 h (T10). mAb B was buffer exchanged using the 5 mL Zeba desalting columns as above prior to isolation of oxidized prepeaks.

2.3.4. Isolation of oxidized mAb variants

The oxidized prepeaks of different mAbs were isolated by mixed mode chromatography under the optimized conditions for each antibody. Isolation of mAb A or B oxidized variants was performed from multiple injections of sample at 500 μg for each injection. mAb C oxidized variants were isolated using PBS buffer [20], with a modification. An additional 160 mM NaCl was added to the mobile phase and the flow rate was set at 0.5 mL/min. Isolated fractions were concentrated and diafiltrated using the Amicon Millipore Ultra (15 mL, 10 kD MWCO) and their respective formulation buffer containing 10 mM methionine. The final protein concentration was determined by UV absorbance at 280 nm using a Nanodrop. The purity of each isolated fraction was determined by re-analysis via mixed mode chromatography.

2.4. Chromatography methods

2.4.1. Size exclusion chromatography at low pH

Size exclusion chromatography (SEC) was performed on the Sepax Technologies SRT or YMC Diol columns using 50 mM sodium acetate, pH 4.9 as mobile phase. The SEC experiments were performed on a Waters Alliance System at room temperature, with wavelength detection at 280 nm, a 0.4 mL/min flow rate, and 80 μg protein injection load.

2.4.2. Mixed mode chromatography

The mixed mode SEC chromatography under high salt conditions was performed with a single or dual buffer system running isocratically using different mobile phases on a Zenix SEC-300MK column (7.8 × 300 nm, 5 μm). Elution was monitored by UV at 280 nm. The mobile phase was phosphate buffered saline (PBS) containing different concentrations of added NaCl, ranging from 0 mM to 2400 mM, dependent on individual antibodies. mAb A was eluted by PBS, pH 7.4 containing 2000 mM NaCl at a flow rate of 0.4 mL/min. The chromatography was run on either an Agilent 1260 series or Waters Alliance or Acquity system with the column temperature of 19 °C. It is worth mentioning that this method is very sensitive to column temperature fluctuations; therefore tight temperature control is critical. For analytical runs, typical injection loads consisted of 50–120 μg antibody. mAb B was eluted using an isocratic gradient consisting of PBS pH 7.4 (buffer A) and PBS containing 4 M NaCl pH 7.4 (buffer B). Elution was developed by increasing the percentage of buffer B until a series of prepeaks and a post peak were resolved completely from the main peak. The optimized elution buffer for mAb B was PBS containing 2400 mM NaCl. For mAb C, due to its intrinsic hydrophobicity, elution was done with PBS as mobile phase without any additional salt. mAb E was eluted with PBS containing 1 M NaCl.

2.4.3. SEC-MALLS

The molecular size analysis of different species separated by the mixed mode chromatography was performed by SEC-MALLS with online Light Scattering HELEOS detector (LS) and differential Refractive Index detector (RI) from Wyatt Technologies as described previously [20–22]. When high salt buffers were used, the protein concentrations of eluting peaks were determined by the UV trace instead of the RI trace. In addition to absorbance at 280 and 214 nm, light scattering and refractive index signals were collected. The molecular mass of detected components was calculated by ASTRA V using dn/dc of 0.185 for all antibodies.

2.4.4. Hydrophobic interaction chromatography

The samples (80 μg each) were separated on column HIC-10 ProPac Analytical Column 4.6 × 100 mm (P/N 063655) equilibrated with 75% mobile phase A (40 mM sodium acetate, 4.0 M sodium chloride, pH 4.9) and 25% mobile phase B (40 mM sodium acetate, pH 4.9). The column temperature was set at 30 °C. Elution of proteins was made at a flow rate of 0.5 mL/min with 25% mobile phase B for 2 min, followed by 100% B for 51 min and then 25% B for 16 min. Chromatography was monitored at 280 nm.

2.5. LC–MS analysis

Fifty microgram of sample was digested in 100 μL with 67 units of Fabricator enzyme for 30 min at 37 °C. Then 25 μL of digested sample (12.5 μg) was mixed with 75 μL 8 M Guanidine-HCl, 5 μL 1 M Tris-HCl, 2 μL DTT and incubated at 56 °C for 20 min shaking at 300 rpm. The digested and reduced sample was injected at 40 μL (5 μg) for LC–MS analysis. The LC–MS experiment was performed on Waters H-class UPLC and Xevo G2 QTOF mass spectrometer. The Agilent ZORBAX 300SB-C8 column (narrow-bore 2.1 × 150 mm 3.5 micron, P.N. 863750-906) was used for reverse phase separation. The sample temperature was set at 5 °C and column temperature was set at 60 °C. The mobile phase A was 0.1% TFA in water, mobile phase B was 99.9% acetonitrile containing 0.1% TFA. Peptides were eluted first with 15% mobile phase B at 0.4 mL/min for 2 min, followed by a gradient of 15–30% mobile phase B for 5 min at 0.2 mL/min and then by a gradient of 30–45% mobile phase B for 15 min, then 88% mobile phase B for 3 min.

The MS front end conditions were set as the following: capillary voltage at 3.0 kV, sampling cone at 35 V, extraction cone at 4.0 V, source temperature at 120 °C, desolvation temperature at 450 °C, cone gas at 30 L/h, desolvation gas at 800 L/h, *m*/*z* range at 600–3800. The mass spectrometry experiment was performed at positive mode and sensitivity mode. The mass spectrometry data was analyzed by using MassLynx software.

3. Results

3.1. Non-specific interactions during size exclusion chromatography on different columns

Size exclusion chromatography (SEC) is a size dependent technique to separate molecules; however, secondary interactions between the eluent and the column resin during SEC have been observed [23]. Although their molecular weights are approximately 148 kD, mAbs A, C, and D showed very different elution times on the Zenix SRT column (Fig. 1A) using 50 mM acetate pH 4.9 as mobile phase. This is consistent with our previous discovery of elution behaviors of different antibodies on the Zenix SEC-300 column [20]. However, these antibodies showed little differences in elution on the YMC diol 200 column (Fig. 1B). Different elution profiles suggest that there were stronger non-specific interactions between antibodies and resin on Zenix or SRT SEC-300MK than on the YMC column. These interactions were most likely hydrophobic in nature as they were enhanced under the high salt concentrations (data not shown). The hydrophobic interactions between the column and antibodies depend on the surface hydrophobic patches of antibodies, and salt, and pH of the mobile phase [24]. Late-eluting antibodies are likely more hydrophobic than early eluting antibodies.

3.2. Analysis of THBP-oxidized mAbs by mixed mode chromatography under different salt conditions

Since Zenix or SRT SEC columns showed strong non-specific interactions, we attempted to take advantage of this property to separate the oxidized variants from unoxidized antibody. mAb C had the longest retention time among tested mAbs, and was separated on the Zenix SEC-300MK column without additional salt in PBS. To generate oxidized variants, the antibody mAb C was treated with *tert*-butyl hydroperoxide (TBHP) which can be used to target exposed methionine residues [25,26]. mAb C, after TBHP treatment, was injected onto Zenix SEC300MK and eluted isocratically. Two new peaks (peaks 1 and 2) were observed before the main peak in mAb C treated samples (1 and 5 h), and the main peak drastically reduced compared to the control (0 h) (Fig. 2A). However, the oxidized species in TBHP-treated mAb A were best separated with PBS containing a high concentration (2.0 M) of NaCl (Fig. 2B). High salts are known to expose hydrophobic patches in proteins creating stronger interaction with the hydrophobic stationary phase [27,28]. Unlike mAb C, no separation for mAb A oxidized species was obtained using PBS as mobile phase (data not shown). The prepeaks progressively increased with longer exposure to TBHP and the main peak decreased concurrently (Fig. 2B and Table 1). In addition, a small amount of prepeaks were also detected in the regular control samples (untreated) when increasing amounts of NaCl was included in PBS (Fig. 2C), likely indicating presence of the oxidized variants in mAb A following purification from the cell culture and storage. The main peak was shifted to later elution with concurrent detection of prepeaks and a post peak. Similarly mAb B prepeaks were resolved on Zenix SEC300MK column using mobile phase consisting of PBS pH 7.4 with increasing concentrations of NaCl (Fig. 2D). The best separation for mAb B was obtained with PBS, pH 7.4 containing 2400 mM NaCl.

Table 1
Quantitation of oxidized species (prepeaks) in mAb A following treatment with TBHP by the mixed mode chromatography.

Sample	Total oxidized prepeaks (%)	Main peak (%)
mAb A control	12.8	87.2
mAb A TBHP 1h	24.7	75.4
mAb A TBHP 2h	37.5	62.5
mAb A TBHP 3h	48.1	51.8
mAb A TBHP 4h	57.1	42.8
mAb A TBHP 6h	74.9	25.2
mAb A TBHP 9h	85.1	14.9

3.3. Analysis of AAPH-oxidized mAbs B and E by a mixed mode chromatography

AAPH, also used to investigate oxidation in therapeutic proteins, can target both methionine and tryptophan residues with higher selectivity for tryptophan [25]. However, extensive exposure to AAPH can lead to covalent aggregate formation via aromatic amino acid linkage such as dityrosine and tryptophan-derived cross-links [29].

Unlike for mAb A (Fig. 2B), TBHP treatment did not increase prepeaks for mAb B in the mixed mode chromatography on the Zenix SEC-300MK (Fig. 3A). This indicates that the mAb B prepeaks do not contain exposed methionine residues, or that methionine oxidized mAb B did not alter its hydrophobicity. However, AAPH treatment progressively increased the prepeaks and decreased the main peak of mAb B over incubation time (Fig. 3B). These results were similar to analytical data by the HIC method (Fig. 3C). This result strongly suggests that the mixed mode method is able to resolve these AAPH induced variants in mAb B. Similarly, the oxidation of mAb E following treatment with AAPH was also demonstrated by the mixed mode chromatography using PBS containing 1 M NaCl as mobile phase (Fig. 3D and Table 2). The oxidized prepeaks clearly increased over time with AAPH.

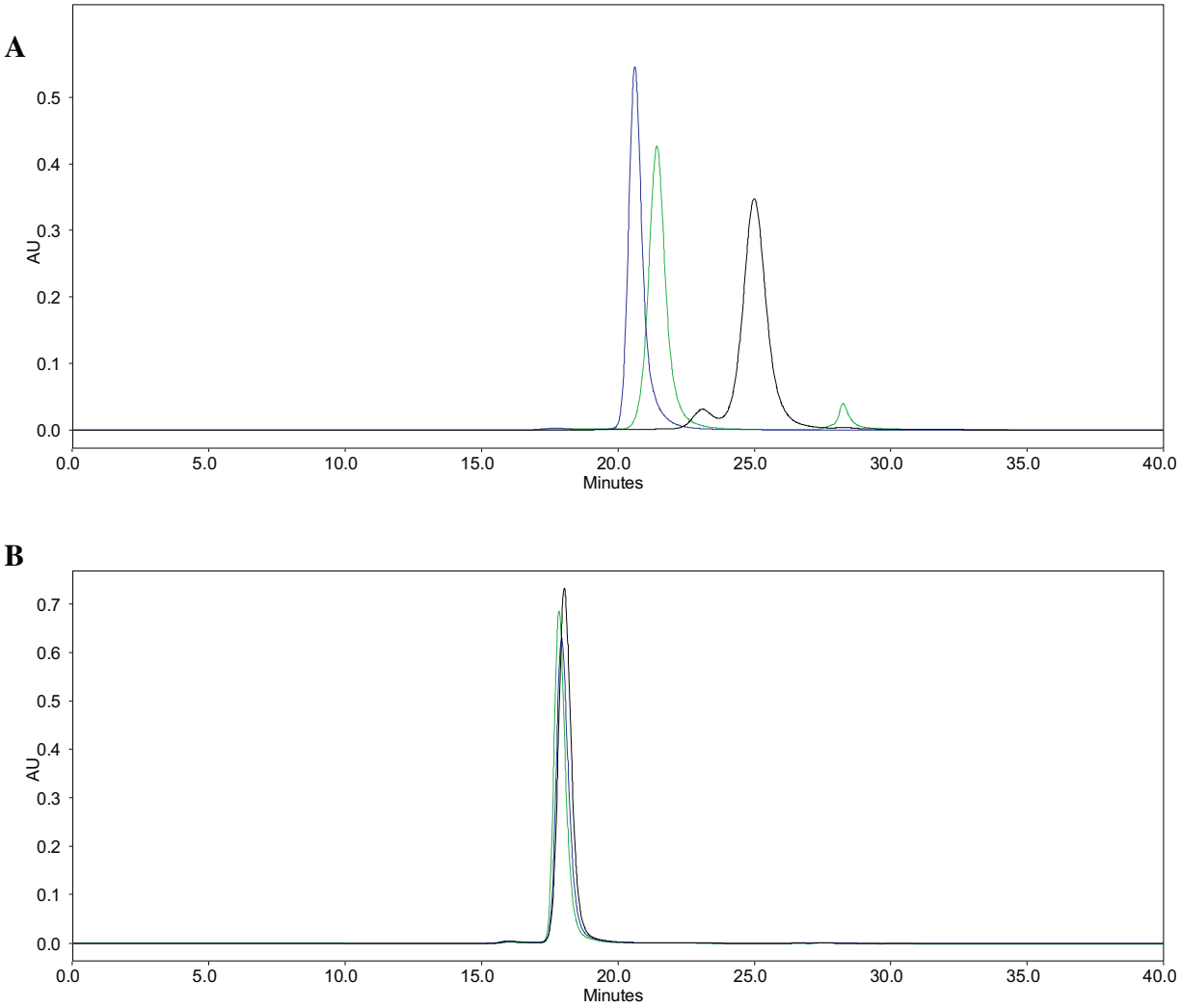


Fig. 1. SEC profiles of mAbs A, C, and D on Sepax SRT SEC-300 column (A) and YMC-Pack Diol 200 column (B) using mobile phase 50 mM acetate pH 4.9 as mobile phase.

Table 2
Quantitation of oxidized species (prepeaks) in mAb E following treatment with AAPH by the mixed mode chromatography.

Sample	Total oxidized prepeaks (%)	Main peak (%)
mAb E AAPH 0h	4.6	95.4
mAb E AAPH 1h	7.9	92.1
mAb E AAPH 3h	10.3	89.7
mAb E AAPH 5h	13.8	86.2
mAb E AAPH 10h	15.9	84.1

3.4. Analysis of photo-stressed mAbs by a mixed mode chromatography

To determine whether the mixed mode chromatography can be also used to monitor the mAb oxidation induced by photo-irradiation, mAb A was stressed under different levels of UV light according to ICH guidelines, and analyzed by the method (Fig. 4). The prepeaks in mAb A increased clearly with the level of photo-irradiation with the concomitant decrease of the main peak, which completely disappeared in 2X ICH light stressed sample. Therefore, the chromatography is equally useful for monitoring antibody oxidation induced by photo-stress.

3.5. Molecular masses of prepeaks in the mixed mode chromatography

To confirm that the prepeaks detected in the mixed mode chromatography did not result from aggregation of antibodies under tested condition, the molecular mass of species present in the prepeaks were determined by the mixed mode SEC chromatography with online multi-angle laser light scattering detector (SEC-MALLS). Results of the online MALLS analysis reveal that major prepeaks, the main peak, and post peak in TBHP treated mAb C (Fig. 5A) and mAb A (Fig. 5B), AAPH-treated mAb B (Fig. 5C), and photo-irradiated mAb A (Fig. 5D) all have a molecular mass in the range of 144–157 kDa, consistent with monomeric antibody. Therefore, major prepeaks did not result from aggregation but represented oxidized monomers. These results reconfirm that under mixed mode conditions the delayed elution of major prepeaks is due to hydrophobic interactions but not due to the size changes.

The dimerized antibody with mass of approximately 300 kDa was detected by SEC-MALLS in the early eluting peaks (16–18 min) in the four hour oxidized AAPH sample or light-stressed sample of mAb A, indicating possible formation of cross-linked species due to ditysorine generated upon extensive exposure to AAPH or photo-irradiation [29,30].

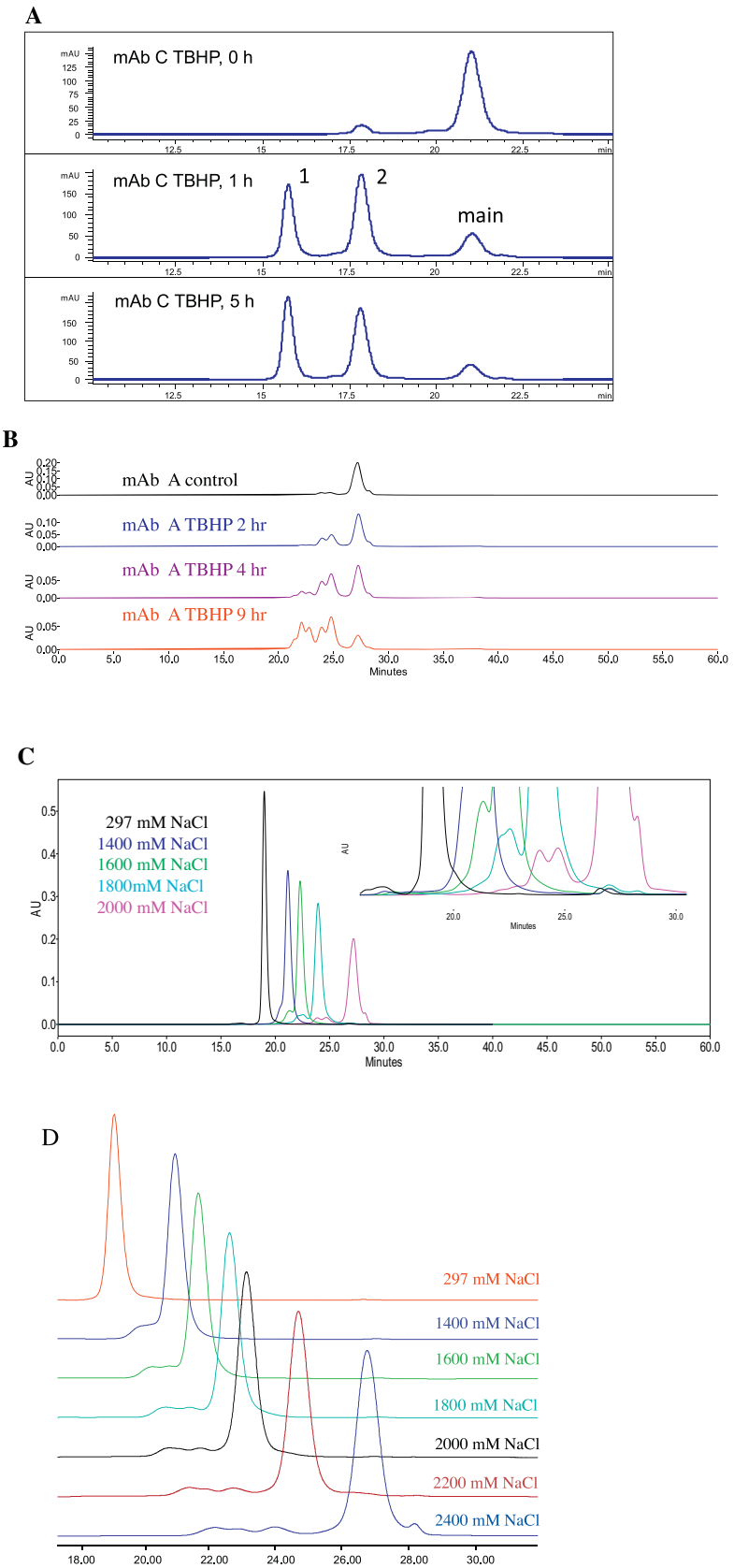


Fig. 2. Separation of mAb C (A) and mAb A (B, C), and mAb B (D) by the mixed mode chromatography on Sepax Zenix SEC-300MK column using PBS containing different concentrations of NaCl as mobile phase. Antibodies were treated with 0.1% TBHP for various times at room temperature. For mAb C, elution was performed with PBS at 0.5 mL/min. For mAb A elution was done with PBS containing 2 M NaCl (B) or increasing concentrations of NaCl (C) at 0.4 mL/min. The control mAbs A and B were separated under different concentrations of NaCl in PBS (C, D). The main peak, prepeaks 1 and 2 are indicated for mAb C.

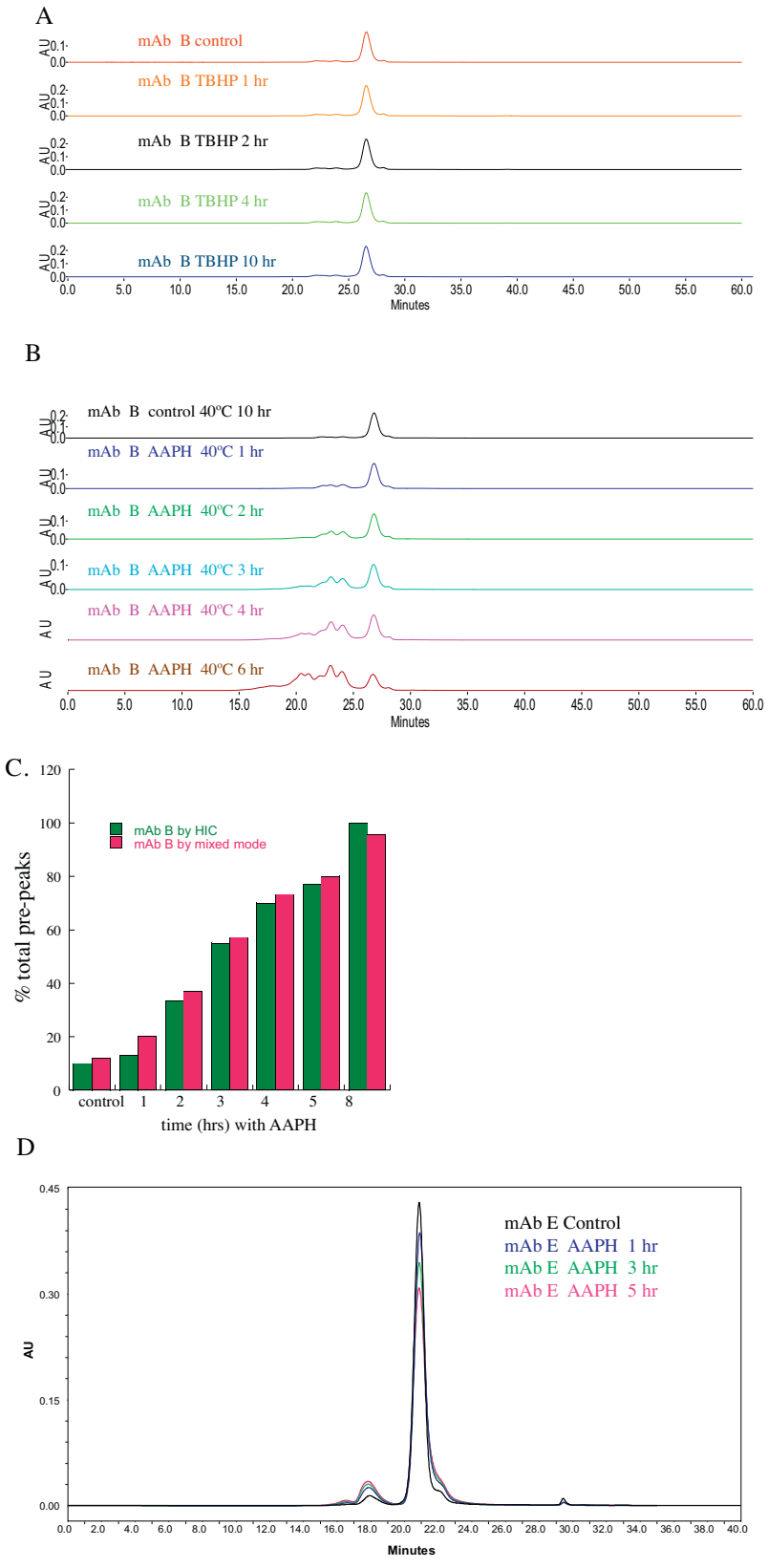


Fig. 3. Analysis for the oxidation of mAb B (5 mg/mL) treated with 0.1% TBHP (A) and 2 mM AAPH (B) and mAb E (D) treated with 2 mM AAPH by the mixed mode chromatography on Zenix SEC-300MK with 2.4 M NaCl (for mAb B) or 1 M NaCl (for mAb E) in PBS pH 7.4. The quantitation of mAb B oxidation by the mixed mode and HIC methods was also compared (C).

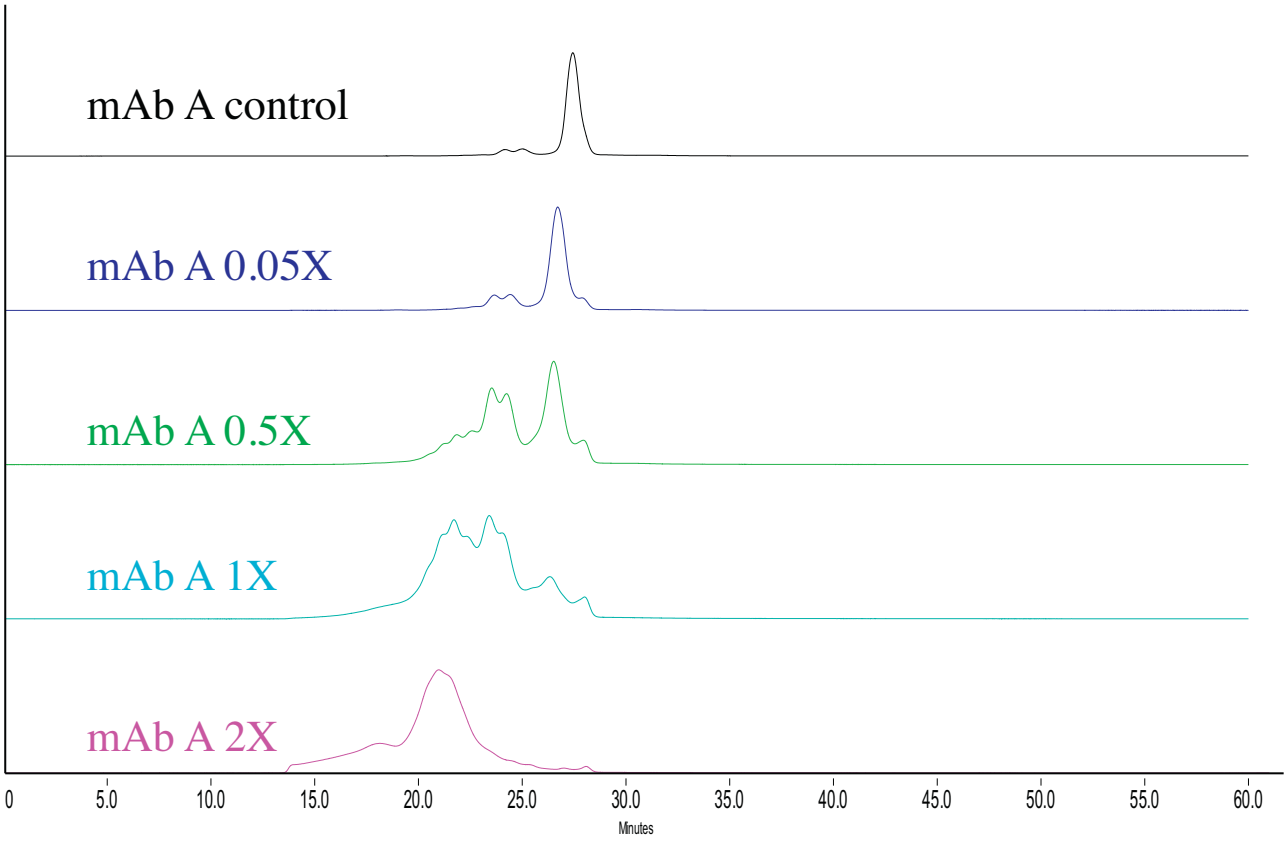


Fig. 4. Analysis of photo-irradiated mAb A by the mixed mode chromatography on Zenix SEC-300 MK. mAb A was photo-irradiated to 0–2X light exposure levels based on ICH guidelines.

Table 3
Quantitation of oxidized species (prepeaks) in mAb B in real time stability studies by the mixed mode chromatography.

Sample	Total oxidized prepeaks (%)	Main peak (%)
mAb B control	17.46	82.53
mAb B 0.5 month 5C	17.08	82.92
mAb B 1.5 months 25C	18.52	81.48
mAb B 3 months 25C	19.98	80.02
mAb B 1 month 40C	25.82	74.18
mAb B 1.5 months 40C	26.69	73.31
mAb B 3 months 40C	41.55	58.46

Table 4
Purity of isolated prepeak fractions from different mAbs.

Sample	Isolated fraction purity (%)	
	Prepeak1	Prepeak2
mAb A control	90%	n.a.
mAb A TBHP 4 h	75%	83%
mAb B control	67%	70%
mAb B AAPH 4 h	83%	86%
mAb C control	74%	n.a.
mAb C TBHP 4 h	n.a.	90%

3.6. Analysis of stability of mAb B by a mixed mode chromatography

To demonstrate the application of the mixed mode chromatography to monitor the real-time stability of antibodies, mAb B was subjected to incubation at different temperatures (5 °C, 25 °C, and 40 °C) and then analyzed by the method (Table 3). The oxidized species were detected as prepeaks and quantitated for each sample. Clearly, mAb B exposed to 25 °C and 40 °C showed increased oxidation levels. The oxidized species increased more significantly at 40 °C than at 25 °C, and up to 41.5% after 3 month incubation.

3.7. Linearity of detection of oxidize species by a mixed mode chromatography

To show the linearity of the detection of the oxidized species, a set of mAb A samples containing different amounts of oxidation, made of different ratios of the control sample and a highly oxidized sample by TBHP, were analyzed by the mixed mode chromatography, and the total prepeak area of each sample was plotted against the expected percentage of oxidized antibody (Fig. 6). The linear response of the prepeak area to the amount of oxidation was clearly observed (Fig. 6 inset plot). In addition, the mixed mode chromatography also showed the good reproducibility (<3% RSD) of detection of oxidized antibody by multiple injections (data not shown).

3.8. Characterization and confirmation of prepeaks as oxidized species in the mixed mode chromatograms by LC–MS

It is very evident from the oxidation kinetic study using TBHP or AAPH as oxidants that the prepeaks separated from the main peak in the mixed mode chromatography likely represents the oxidized antibody, as they increased over time of treatment with TBHP, AAPH, or photo-irradiation, and had a molecular mass similar to the monomer. To unequivocally confirm this, prepeaks were purified and then subjected to LC–MS analysis for confirmation of oxidation. The purity of the isolated fractions was determined by reinjection on the Zenix SEC-300MK column, and ranged from 67% to 90% (Table 4).

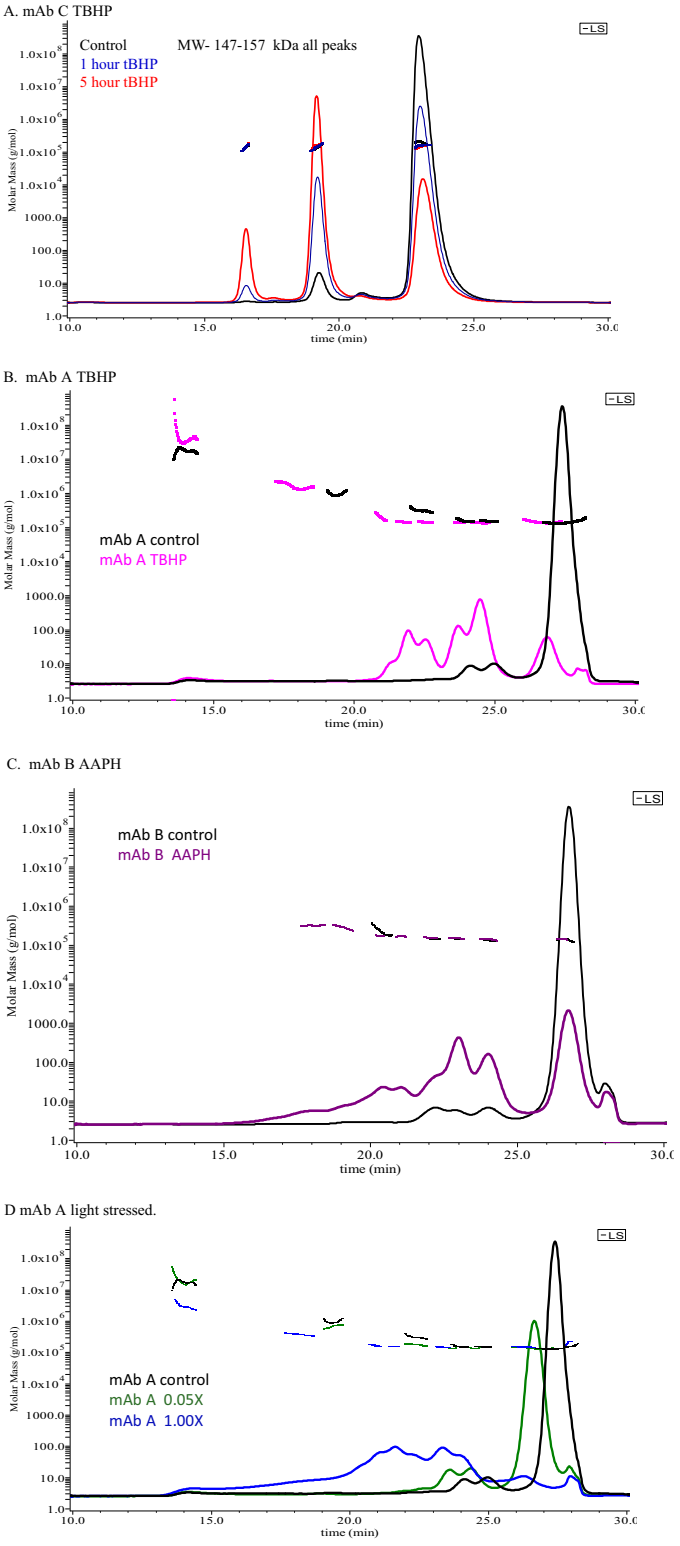


Fig. 5. Analysis of the molecular weights of the prepeaks and main peaks of mAbs C and A during the mixed mode chromatography by SEC-MALLS. (A) mAb C oxidized by TBHP, (B) mAb A oxidized by TBHP, (C) mAb B oxidized by AAPH, and (D) mAb A treated by photo-irradiation at different light exposure levels. Light scattering overlay and mass distribution across the peaks were shown for each mAb during the mixed mode chromatography on Zenix SEC-300MK using PBS (for mAb C) or PBS containing 2.0 M NaCl (for mAb A) or 2.4 M NaCl (for mAb B) as mobile phase.

3.8.1. *mAb A*

Both prepeaks and main peak of mAb A were purified from the unstressed control or TBHP-treated sample by the mixed mode chromatography, and then were digested with Fabricator, a specific enzyme that cleaves at the hinge of the antibody to generate (Fab) 2 and Fc fragments [31]. The digests were then analyzed by LC–MS following denaturing and reduction (Fig. 7A and Table 5). The Fabricator-digested main peak was resolved by RP-HPLC into three major peaks, corresponding to fragment Fc (16 min), light chain (LC) (18.6 min), and fragment Fd (22 min), and one minor peak

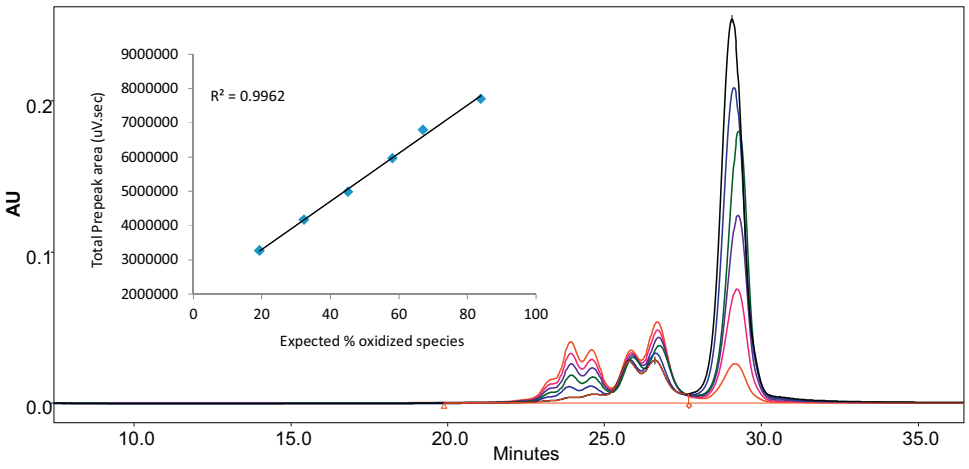


Fig. 6. The linearity of detection for oxidized antibody species by the mixed mode chromatography. The mAb A samples containing different amounts of oxidized species, made of different ratios of the control sample and a highly oxidized sample by TBHP were analyzed.

at 21 min. Fd represents the N-terminal fragment of HC, and Fc is the C-terminal fragment of HC after the cleavage. The isolated prepeaks from the mixed mode chromatography however displayed a much higher amount of the minor peak at 21 min on RP-HPLC, corresponding to oxidized Fd (Fd oxi), with a mass 16 Da larger than unoxidized Fd (Table 5). More oxidized Fd was detected in the prepeaks (prepeaks 1 and 2) of TBHP-treated mAb A. In addition, oxidized Fc fragments (Fc, oxi1 and Fc, oxi2) with masses of 16 and 32 Da larger than unoxidized Fc mass were detected in the mixed mode prepeaks of the TBHP-treated mAb A. Fd oxi was present at a higher amount in prepeak 2, likely representing antibodies with oxidization occurring on both arms. Therefore, the data here clearly confirmed the prepeaks from the mixed mode chromatography as the oxidized species of the antibody.

3.8.2. *mAb B*

Similarly, Fd oxi fragments eluting at approximately 19.5, 20.0, and 20.1 min were detected in the mixed mode prepeak 1 and 2 of the unstressed control sample, and at a much higher level in prepeaks 1 and 2 of the AAPH treated samples (Fig. 7B). The Fd oxi fragments were barely detected in the main peak of the unstressed control. However, the prepeak 2 from the control contained an additional peak at 19.2 min, which is the mixture of HC 77–397, G0 F and HC 72–397, G0F, likely representing aberrantly fragmented peptides. Like in the case of mAb A, oxidized Fc fragments (Fc oxi1 and Fc oxi2) with masses 16 and 32 Da higher than unoxidized Fc mass were detected in the prepeaks of the AAPH-treated mAb B (Table 6). Therefore, the prepeaks resolved by the mixed mode chromatography are the oxidized variants of the antibody.

3.8.3. *mAb C*

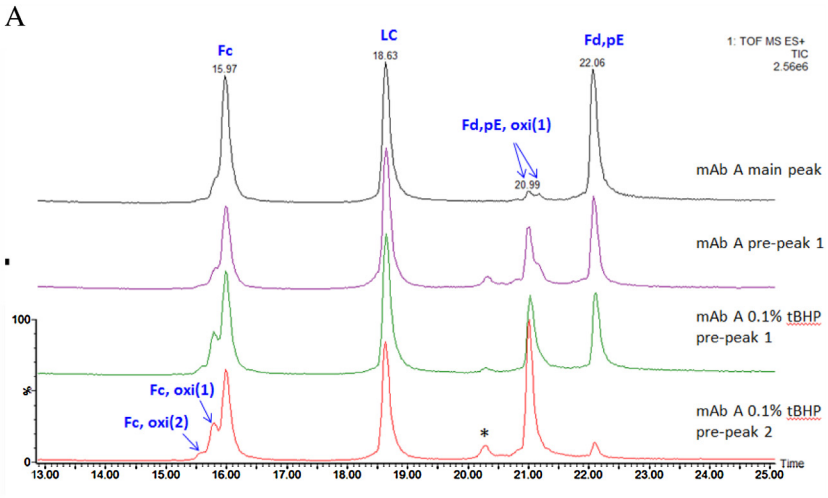
mAb C is known to have a Met in one of CDRs that is prone to oxidation. Fabricator digest of purified mixed mode prepeaks eluted in RP-HPLC in the order of Fc, Fd, and LC fragments (Fig. 7C). Fd oxi 1 fragment eluting at approximately 19.1 min was clearly detected in prepeaks 1 and 2 of the unstressed control sample, and at a much higher level in prepeak 2 of the TBHP-treated sample. Fd oxi:Fd ratios were approximately 1:1 in prepeak 1, representing one Fab arm containing oxidized Met; the ratio increased in prepeak2, corresponding to both arms containing oxidized Met (Fig. 7C and Table 7, and data not shown). These Fd oxi fragments were barely detected in the main peak of the unstressed control. In TBHP-treated mAb C, almost all Fd was converted into Fd oxi, reflecting that Met on both Fab arms was oxidized. Oxidized Fc fragment Fc oxi1 with increased mass of 16 Da was detected in the prepeaks of the unstressed control and the prepeak 2 of TBHP-treated mAb C. Therefore, the prepeaks resolved by the mixed mode chromatography correspond to the oxidized variants of mAb C.

4. Discussion

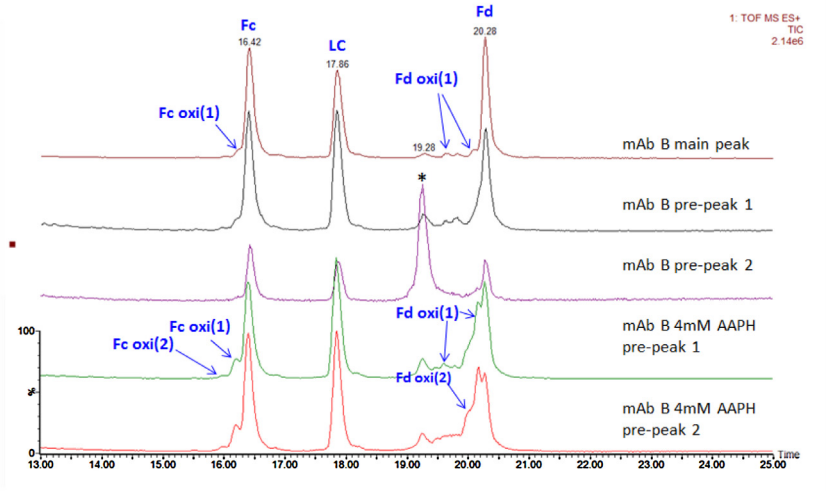
The consequence of oxidation could potentially result in the loss of antigen binding affinity, depending on the location of oxidized amino acid. Methionine residues in CDR are often found to be oxidized since they are well exposed on antibody surfaces to oxidants. In many cases, oxidation of proteins can lead to changes in local conformation, and in particular, in the surface hydrophobicity. Oxidized amino acid residues become more hydrophilic and reduce the hydrophobicity, thus alter the interaction with the nearby residues,

Table 5
LC–MS analysis of Fabricator-digested main and prepeaks from the mixed mode chromatography of mAb A control and TBHP-treated samples.

RT (min)	Fragment	Theoretical mass (Da)	Control (Da)		TBHP-treated (Da)	
			Prepeak1	Main peak	Prepeak1	Prepeak2
15.7	Fc: H242-452 (-K), G0F, oxi (2)	25,268			25,268	25,268
	Fc: H242-452 (-K), G1F, oxi (2)	25,430				25,430
15.8	Fc: H242-452 (-K), G0F, oxi (1)	25,252	25,252	25,252	25,252	25,253
	Fc: H242-452 (-K), G1F, oxi (1)	25,414	25,416	25,414	25,414	25,414
	Fc: H242-452, G0F	25,364	25,366	25,364	25,366	25,364
	Fc: H242-452, G1F	25526	25,527	25,526		
16.0	Fc: H242-452 (-K), G0F	25,236	25,236	25,236	25,236	25,236
	Fc: H242-452 (-K), G1F	25,398	25,398	25,398	25,398	25,398
16.4	Fc: H242-452 (-K), G0F, deami-(2)	25,238			25,238	25,236
18.6	LC	23,278	23,278	23,278	23,278	23,278
21.0	Fd: H1-241 (pE), oxi (1)	25,770	25,770	25,770*	25,770	25,770
22.1	Fd: H1-241 (pE)	25,754	25,754	25,754	25,754	25,754



*: Mixed MS spectra with high noise



*: mixture of H115-436, H115-444, and H5-332

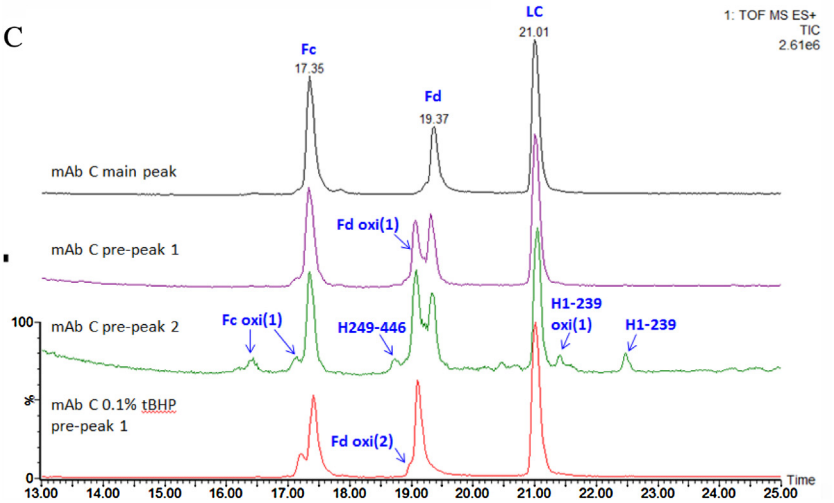


Fig. 7. LC–MS analysis for purified mAb A (A), mAb B (B) and mAb C (C) prepeaks and main peak from the mixed mode chromatography. The prepeaks and main peaks purified were digested with Fabricator, and analyzed by LC–MS following denaturing and reduction.

Table 6
LC–MS analysis of Fabricator-digested main and prepeaks from the mixed mode chromatography of mAb B control and AAPH-treated samples.

RT (min)	Fragment	Theoretical. mass (Da)	Control (Da)			AAPH-treated (Da)	
			Main peak	Prepeak1	Prepeak2	Prepeak1	Prepeak2
16.0	Fc: HC239-446, (-K), G0F, oxi(2)	25,268				25,269	25,267
16.2	Fc: HC239-446, (-K), G0F, oxi(1)	25,252	25,251	25,251		25,252	25,252
	Fc: HC239-446, (-K), G1F, oxi(1)	25,414	25,414	25,412		25,414	25,414
16.4	Fc: HC239-446, (-K), G0F	25,236	25,236	25,236	25,237	25,236	25,236
	Fc: HC239-446, (-K), G1F	25,398	25,399	25,398	25,398	25,399	25,398
17.8	LC	23,429	23,429	23,429	23,429	23,429	23,429
19.25	HC 77-397, G0F	36,648		36,646	36,647	36,644	36,646
	HC 72-397, G0F	37,148		37,150	37,148	37,147	37,145
19.79	Fd: HC1-238, oxi(1)	25,484	25,484	25,485	25,485	25,484	25,485
20.06	Fd: HC1-238, oxi(2)	25,500					25,501
20.16	Fd: HC1-238, oxi(1)	25,484	25,485	25,484		25,485	25,485
20.26	Fd: HC1-238	25,468	25,469	25,469	25,469	25,469	25,469

Table 7
LC–MS analysis of Fabricator-digested main and prepeaks from the mixed mode chromatography of mAb C control and TBHP-treated samples.

RT (min)	Fragment	Theoretical mass (Da)	Control			TBHP-treated
			Main peak	Prepeak1	Prepeak2	Prepeak2
16.4	Fc: HC 237-446,-K, G0F, oxi(1)	25,236			25,237	
17.1	Fc: HC 237-446,-K, G0F, oxi(1)	25,236	25,236	25,236	25,237	25,236
	Fc: HC 237-446,-K, G1F, oxi(1)	25,398	25,399			25,398
17.4	Fc: HC 237-446,-K, G1F	25,382	25,382	25,383		25,383
	Fc: HC 237-446,-K, G0F	25,220	25,220	25,220	25,220	25,220
18.7	HC 249-446, -K, G0F	23,924			23,926	
18.96	Fd: HC 1-236, oxi(2)	25,548				25,547
19.1	Fd: HC 1-236, oxi(1)	25,530		25,530	25,530	25,530
19.4	Fd: HC 1-236	25,514	25,514	25,514	25,514	
21.0	LC	23,744	23,745	23,744	23,745	23,745
21.4	HC 1-239, oxi(1)	25,771			25,769	
22.5	HC 1-239	25,755			25,754	

leading to conformational changes at either the local or global levels of overall structures. Any separation techniques that rely on hydrophobicity as the main mechanism for resolving the oxidized and native proteins would provide some success in analysis of oxidation. Hydrophobic interaction chromatography (HIC) [16], reverse phase-HPLC coupled with Fabricator digestion [31], and even Protein A chromatography [17] have been utilized for protein oxidation analysis using the hydrophobic property of mAbs. These methods have suffered problems with limited resolution for different oxidized variants or with long analytical time.

Size exclusion chromatography (SEC) is a universal technique to separate and analyze sizes of biologics molecules on a silica based column with an aqueous and native buffer containing appropriate salts as the mobile phase. Normally separation during SEC occurs based on the size or hydrodynamic radius of molecules to be analyzed, with minimal secondary or non-specific interactions between the analyte and resin. However, undesired non-specific interactions could occur between SEC columns and analytes under proper conditions. We discovered surprisingly that antibodies displayed very distinct retention times on Sepax Zenix or SRT SEC300 columns in spite of the fact that tested mAbs had very similar molecular sizes. This indicates the possibility of interactions between different hydrophobic patches on the individual antibodies and the column resin. Although this hydrophobic interaction is undesirable for regular SEC analysis, in this paper we explored this otherwise undesirable interaction to analyze antibody oxidation.

Our results clearly showed that the oxidized species of different mAbs were well separated from the main monomer species as prepeaks by the mixed mode chromatography on Zenix SEC-300MK column using PBS containing NaCl at a concentration ranging 0–2.4 M specific to the tested antibody. The separation allows quantitative analysis of oxidation levels in antibody samples. The non-specific interactions occurring on the Zenix SEC column are likely hydrophobic, which can be enhanced by increasing the salt concentration in the mobile phase. The high salts expose hydrophobic patches in proteins creating stronger interaction with the hydrophobic stationary phase [27,28].

The mixed mode conditions used depended on the surface hydrophobicity of the antibodies, which were tested on Zenix SEC-300MK with PBS as mobile phase. The condition for the optimal separation of oxidized species from the main peak can be determined by running PBS containing different NaCl concentrations. This can be readily performed by running different % of PBS (mobile phase A) and PBS with 4 M NaCl (mobile phase B), automated by the software. mAb C eluted much later than mAbs A and B in the regular SEC assays on Sepax SRT SEC-300 column, suggesting that mAb C has more hydrophobic surface than mAbs A and B. Therefore, mAb C can be analyzed for oxidation simply using PBS as mobile phase, while mAbs A and B need to be analyzed under a high salt condition for oxidized species. Apparently, antibodies are separated under the mixed mode conditions by both size and hydrophobicity of the antibody molecules. It should be noted that any SEC columns that display the strong hydrophobic interactions should be avoided in normal antibody SEC. However, another version of Zenix SEC column (Zenix SEC-300 without MK in the catalog number) with much less hydrophobic interactions should be used in this case.

When a sample is extensively oxidized by oxidants or conditions, such as AAPH and photo-irradiation, some of the oxidized antibody is expected to form aggregates due to the cross-linking of oxidized molecule. These aggregates are expected to elute earlier than oxidized monomers and not to impact quantitation of the overall oxidation in the sample. Unoxidized aggregates could co-elute with major oxidized prepeaks during the mixed mode chromatography, but are present at a low level that is not expected to affect the oxidation analysis. However, besides the surface hydrophobicity of the antibodies, the column consistency achieved

via column screening is critical for maintaining a robust mixed chromatography.

Multiple prepeaks resolved in the mixed mode chromatography indicate the heterogeneity of the oxidized antibody, containing different oxidation sites. Therefore, the chromatography can be used to monitor not only the overall oxidation, but also the individual oxidation events at different sites. The conditions used for our mixed mode chromatography are considered non-denaturing, and allow facile isolation of oxidized variants for further biochemical and biological characterization such as potency assays, and oxidation site identification by other analytical techniques. Unlike HIC, this method also allows in-line characterization of molecular masses of the species to distinguish the aggregates and oxidized variants in the prepeaks. In summary, this simple mixed mode chromatography is useful as a complement method to HIC assays for monitoring mAb oxidization during the development and stability studies without the dependence on the time consuming LC–MS methods.

5. Conclusions

A mixed mode chromatography method was developed that separates oxidized hydrophilic variants (oxidized prepeaks) from the main peak of the respective IgG molecules. The method utilized the unique mixed mode property of Sepax Technologies Zenix SEC-300MK column using PBS, pH 7.4 containing antibody-specific concentrations of NaCl as mobile phase to separate the oxidized species at 19 °C. The ability to measure methionine and tryptophan oxidation with the final mixed mode chromatography was confirmed with online SEC-MALLS analysis and LC–MS. The mixed mode method has been applied to analyze our force degraded samples, and routine samples for oxidization levels.

Acknowledgements

We want to thank Steve Farrand for his support, and Dr. Robin Ehrick for reviewing the manuscript.

References

[1] L. Zitvogel, G. Kroemer, Cancer: antibodies regulate antitumour immunity, Nature 521 (2015) 35–37.
[2] J.M. Redman, E.M. Hill, D. AlDeghaither, L.M. Weiner, Mechanisms of action of therapeutic antibodies for cancer, Mol. Immunol. 67 (2015) 28–45.
[3] O.H. Brekke, I. Sandlie, Therapeutic antibodies for human diseases at the dawn of the twenty-first century, Nat. Rev. Drug Discov. 2 (2003) 52–62.
[4] K. Imai, A. Takaoka, Comparing antibody and small-molecule therapies for cancer, Nat. Rev. Cancer 6 (2006) 714–727.
[5] D.S. Chen, I. Mellman, Oncology meets immunology: the cancer-immunity cycle, Immunity 39 (2013) 1–10.
[6] H. Liu, G. Gaza-Bulseco, D. Faldu, C. Chumsae, J. Sun, Heterogeneity of monoclonal antibodies, J. Pharm. Sci. 97 (2008) 2426–2447.
[7] N. Jenkins, L. Murphy, R. Tyther, Post-translational modifications of recombinant proteins: significance for biopharmaceuticals, Mol. Biotechnol. 39 (2008) 113–118.
[8] A. Bertolotti-Ciarlet, W. Wang, R. Lownes, P. Pristatsky, Y. Fang, T. McKelvey, Y. Li, Y. Li, J. Drummond, T. Prueksaritanont, J. Vlasak, Impact of methionine oxidation on the binding of human IgG1 to Fc Rn and Fc gamma receptors, Mol. Immunol. 46 (2009) 1878–1882.
[9] H. Pan, K. Chen, L. Chu, F. Kinderman, I. Apostol, G. Huang, Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn, Protein Sci. 18 (2009) 424–433.
[10] Z. Wei, J. Feng, H.Y. Lin, S. Mullapudi, E. Bishop, G.I. Tous, J. Casas-Finet, F. Hakki, R. Strouse, M.A. Schenerman, Identification of a single tryptophan residue as critical for binding activity in a humanized monoclonal antibody against respiratory syncytial virus, Anal. Chem. 79 (2007) 2797–2805.
[11] W. Wang, J. Vlasak, Y. Li, P. Pristatsky, Y. Fang, T. Pittman, J. Roman, Y. Wang, T. Prueksaritanont, R. Ionescu, Impact of methionine oxidation in human IgG1 Fc on serum half-life of monoclonal antibodies, Mol. Immunol. 48 (2011) 860–866.
[12] J. Mo, A.A. Tymiak, G. Chen, Structural mass spectrometry in biologics discovery: advances and future trends, Drug Discov. Today 17 (2012) 1323–1330.
[13] G. Chen, B.M. Warrack, A.K. Goodenough, H. Wei, D.B. Wang-Iverson, A.A. Tymiak, Characterization of protein therapeutics by mass spectrometry: recent developments and future directions, Drug Discov. Today 16 (2011) 58–64.
[14] I.A. Kaltashov, C.E. Bobst, R.R. Abzalimov, G. Wang, B. Baykal, S. Wang, Advances and challenges in analytical characterization of biotechnology products: mass spectrometry-based approaches to study properties and behavior of protein therapeutics, Biotechnol. Adv. 30 (2012) 210–222.
[15] M. Haverick, S. Mengisen, M. Shameem, A. Ambrogelly, Separation of mAbs molecular variants by analytical hydrophobic interaction chromatography HPLC: overview and applications, mAbs 6 (2014) 852–858.
[16] D. Boyd, T. Kaschak, B. Yan, HIC resolution of an IgG1 with an oxidized Trp in a complementarity determining region, J. Chromatogr. B Analyt. Technol. Biomed. Life Sci. 879 (2011) 955–960.
[17] C. Loew, C. Knoblich, J. Fichtl, N. Alt, K. Diepold, P. Bulau, P. Goldbach, M. Adler, H.C. Mahler, U. Grauschopf, Analytical protein a chromatography as a quantitative tool for the screening of methionine oxidation in monoclonal antibodies, J. Pharm. Sci. 101 (2012) 4248–4257.
[18] C. Wong, C. Strachan-Mills, S. Burman, Facile method of quantification for oxidized tryptophan degradants of monoclonal antibody by mixed mode ultra performance liquid chromatography, J. Chromatogr. A 1270 (2012) 153–161.
[19] X. Yang, A. Ambrogelly, Enlarging the repertoire of therapeutic monoclonal antibodies platforms: domesticating half molecule exchange to produce stable IgG4 and IgG1 bispecific antibodies, Curr. Opin. Biotechnol. 30 (2014) 225–229.
[20] X. Yang, Y. Zhang, F. Wang, L.J. Wang, D. Richardson, M. Shameem, A. Ambrogelly, Analysis and purification of IgG4 bispecific antibodies by a mixed-mode chromatography, Anal. Biochem. 484 (2015) 173–179.
[21] P. Orth, L. Xiao, L.D. Hernandez, P. Reichert, P.R. Sheth, M. Beaumont, X. Yang, N. Murgolo, G. Ermakov, E. DiNunzio, F. Racine, J. Karczewski, S. Secore, R.N. Ingram, T. Mayhood, C. Strickland, A.G. Therien, Mechanism of action and epitopes of *Clostridium* difficile toxin B-neutralizing antibody bezlotoxumab revealed by X-ray crystallography, J. Biol. Chem. 289 (2014) 18008–18021.
[22] X. Mou, X. Yang, H. Li, A. Ambrogelly, D.J. Pollard, A high throughput ultra performance size exclusion chromatography assay for the analysis of aggregates and fragments of monoclonal antibodies, Pharm. Process. 2 (2014) 141–156.
[23] P. Hong, S. Koza, E.S. Bouvier, Size-exclusion chromatography for the analysis of protein biotherapeutics and their aggregates, J. Liq. Chromatogr. Relat. Technol. 35 (2012) 2923–2950.
[24] N.P. Golovchenko, I.A. Kataeva, V.K. Akimenko, Analysis of pH-dependent protein interactions with gel filtration medium, J. Chromatogr. 591 (1992) 121–128.
[25] J.A. Ji, B. Zhang, W. Cheng, Y.J. Wang, Methionine, tryptophan, and histidine oxidation in a model protein, PTH: mechanisms and stabilization, J. Pharm. Sci. 98 (2009) 4485–4500.
[26] J.W. Chu, J. Yin, D.I. Wang, B.L. Trout, A structural and mechanistic study of the oxidation of methionine residues in hPTH(1-34) via experiments and simulations, Biochemistry 43 (2004) 14139–14148.
[27] Pahlman, J. Rosengren, S. Hjerten, Hydrophobic interaction chromatography on uncharged sepharose derivatives. Effects of neutral salts on the adsorption of proteins, J. Chromatogr. 131 (1977) 99–108.
[28] J.A. Queiroz, C.T. Tomaz, J.M. Cabral, Hydrophobic interaction chromatography of proteins, J. Biotechnol. 87 (2001) 143–159.
[29] R. Torosantucci, C. Schoneich, W. Jiskoot, Oxidation of therapeutic proteins and peptides: structural and biological consequences, Pharm. Res. 31 (2014) 541–553.
[30] A. Arenas, C. Lopez-Alarcon, M. Kogan, E. Lissi, M.J. Davies, E. Silva, Chemical modification of lysozyme, glucose 6-phosphate dehydrogenase, and bovine eye lens proteins induced by peroxyl radicals: role of oxidizable amino acid residues, Chem. Res. Toxicol. 26 (2013) 67–77.
[31] Y. An, Y. Zhang, H.M. Mueller, M. Shameem, X. Chen, A new tool for monoclonal antibody analysis: application of IdeS proteolysis in IgG domain-specific characterization, mAbs 6 (2014) 879–893.