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Characterization and monitoring of charge variants of a recombinant monoclonal antibody using microfluidic capillary electrophoresis-mass spectrometry

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ABSTRACT

Rigorous characterization of biotherapeutics, and monoclonal antibodies in particular, is a challenging task in terms of ensuring safety, efficacy, and potency of a therapeutic agent because of structural heterogeneity during cell culture, purification and storage. In this work, we used microfluidic capillary electrophoresis-mass spectrometry to analyze intact monoclonal antibody and assess the root cause of increases in acidic and basic variants under stress at high temperature. The antibody was analyzed at multiple levels, including its intact state under native conditions, and subunit and peptide levels. The normal and degraded antibodies at different time points were characterized and compared with each other. We concluded that the basic variants in the unstressed sample were produced C-terminal amidation, while the acidic variants were produced by deamidation. In stressed samples, change in the acidic and main peaks were caused by deamidation, and changes in the basic peaks were caused by both deamidation and oxidation. These results demonstrate that microfluidic capillary electrophoresis-mass spectrometry (CE-MS) is a powerful direct and generic tool for separation and identification of charge heterogeneity of biotherapeutics.

1. Introduction

In 2019, there were16,181 drugs in the research and development stage, and approximately 40% of these were biological drugs, which was a large increase on their proportion of 15% in 1995 [1]. The discovery and development of monoclonal antibody (mAb) therapeutics is resource demanding and technically challenging. Specifically, it has been well recognized for the challenges associated with Chemistry, Manufacturing and Controls (CMC) development including high aggregation, high viscosity and susceptibility to chemical degradation and insufficient product stability have been commonly. Conventionally, selection of mAb candidate from the early discovery to development stage uses limited criteria such as antigen binding and *in vivo* properties related to the safety, pharmacokinetics (PK) and pharmacodynamics (PD) in animal models [2]. Because mAbs are large proteins, their development for therapeutic use in humans poses many challenges throughout the entire process [3]. Characterization and understanding of degradation pathways plays an important role in ensuring product integrity and manufacturing consistency. During the manufacturing process, different types of microheterogeneity can be introduced either because of enzymatic processing or spontaneous degradation. These variants need to be characterized and suitable formulations need to be developed to minimize further degradation of the purified antibody during storage [4].

Analysis of charge heterogeneity is an important method for monitoring mAbs. Numerous variants are commonly observed when mAbs are analyzed by charge-based separation techniques such as isoelectric focusing (IEF) gel electrophoresis, capillary IEF (cIEF) gel electrophoresis, imaged cIEF (icIEF) gel electrophoresis [5], cation exchange chromatography (CEX), anion exchange chromatography (AEX) [6], cIEF-mass spectrometry (MS) [7] and capillary electrophoresis (CE)-MS [8]. Modifications that result in formation of acidic variants include deamidation, sialic acid, methionine oxidation, asp isomerization, intermediate succinimide and trisulfide bond. Formation of basic variants

Abbreviations: BGE, background electrolyte; CE, capillary electrophoresis; cIEF, capillary isoelectric focusing; CEX, cation exchange chromatography; IEF, isoelectric focusing; MS, mass spectrometry; mAb, monoclonal antibody; UV, ultraviolet.

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involves C-terminal Lys clipping, C-terminal amidation, leader sequence, incomplete disulfide bond, methionine oxidation [2]. CEX is widely used in the biopharmaceutical industry to monitor charge heterogeneity in proteins and for separation of protein variants, including deamidated species [9–14]. However, characterization of the peaks separated by CEX is challenging because of the complexity of charge heterogeneity associated with mAbs. Purified CEX fractions have to be collected and analyzed by peptide mapping, size exclusion chromatography (SEC), intact and reduced-alkylated reversed phase techniques. Biophysical characterization, IEF and isoquant analysis are also performed to determine the nature of degraded proteins [15]. The conventional methods currently used for identification of CEX peaks involve collection of fractions and are time-consuming. In addition, because they involve indirect characterization, these methods may introduce false positives [16]. Traditional methods involve collection of peaks to analyze the charge isomerism of antibodies [17]. These methods include CEX-MS [8,18], imaged cIEF-MS [7], and CE-MS [19].

In this paper, CE-MS was used to identify mAb charge variants. Compared with other technologies with different separation principles, CE-MS involves minimal sample preparation, is rapid, and only uses a small amount of sample (nanogram scale). Characterization and analysis were carried out for stressed samples. This article introduces a simple and straightforward microfluidic CE-MS workflow for characterization of acidic and basic variants of mAbs at intact protein, subunit, and peptide levels. The root cause of the increase in acidic and basic variants was explored in stability experiments.

2. Materials and methods

2.1. Materials and reagents

The mAb canonical IgG1 was manufactured by HJB Ltd. The mAb contained two copies of a light chain, each containing a variable domain (VL) and a constant domain (CL), and two copies of a heavy chain, each containing a variable domain (VH), three constant domains (CH1, CH2, and CH3), with a hinge region between CH1 and CH2. The two heavy chains were disulfide bonded to each other via two cysteines in the hinge region, and the light chain and heavy chain were connected by a disulfide bond between CL and CH1. The formulation buffer (pH 6.0) contained 20 mM histidine, 7.5% sucrose, and 0.05% polysorbate 80 (PS80), and the concentration of antibody in the formulation buffer was 30 mg/mL. Dithiothreitol (DTT, catalog# D0632-25G), immunoglobulin G-degrading enzyme of *Streptococcus pyogenes* (IdeS) (catalog# V7511) and a Rapid Digestion-Trypsin/Lys-C Kit (catalog# VA1061) were all obtained from Promega (Madison, WI, USA). Iodoacetamide (IAM catalog# I1149-25G) was purchased from Sigma-Aldrich (St. Louis, MO, USA). Microfluidic CE separation was carried out on a ZipChi platform (908 Devices Inc., Boston, MA, USA) using an HRN (high resolution native) microchip and the BGE (background electrolyte) provided in the Native antibody Kit (catalog# 850-00048, 908 Devices Inc., Boston, MA, USA), for native MS analysis. The BGE provided with Native antibody kit is a proprietary formulation based on ammonium acetate at pH 5.5 and modified with 4% DMSO. A ZipChip peptides assay kit (catalog# 850-00034) and HR (high-resolution) chip (catalog# 810-00194) were used for peptide mapping analysis. A CEX column ProPac WCX-10 (catalog# 302972, Thermo Fisher Scientific (China), Shanghai, China) was used for the CEX experiment.

2.2. Sample preparation

An antibody sample with concentration of 30 mg/mL was first diluted to 5 mg/mL using ultrapure water. Two stressed mAbs were prepared by storing them at 40 °C for 2 weeks and 4 weeks in the dark. Then the samples were stored at 2°C-8°C until required for analysis. For Native CE-MS analysis of intact antibody, an antibody sample was further diluted to 0.5 mg/mL using the diluent from the Native Antibodies kit. For analysis of the antibody subunit, the sample was diluted to 0.5 mg/mL with ultrapure water, then 1 μL of IdeS (50 units/μL) was added and the solution was mixed well. The IdeS solution was prepared by adding 100 μL of ultrapure water to a bottle containing 5000 units of IdeS. Enzyme was added to 50 μL of the sample solution, which was then incubated at 37 °C for 30 min. Next, 5 μL of DTT (1 M) was added and the solution was incubated at 37 °C for 30 min. Finally, 50 μL ultrapure water was added to give a sample solution with a concentration of approximately 0.25 mg/mL. For peptide mapping experiments, samples were diluted to 1 mg/mL in Rapid Digest Buffer from the Rapid Digestion-Trypsin/Lys-C Kit. Then, 50 μL of protein (50 μg) as substrate was added to 150 μL of Rapid Digest Buffer. Protease (100 μg, Rapid Trypsin/Lys-C Mix) was resuspended in 100 μL of Resuspension Buffer from the kit to give a concentration of 1 mg/mL. Next, 5 μL of the resuspended Rapid Trypsin/Lys-C Mix was added to the sample solution in a 1:10 enzyme/substrate ratio. The solution was incubated for up to 60 min at 70 °C. After digestion, the peptides were reduced in 1 M DTT at 50 °C for 30 min and alkylated in 1 M iodoacetamide for 30 min in the dark at room temperature. The reaction was terminated by adding 5 μL of formic acid for every 200 μL of the digestion reaction solution. The solution was centrifuged for 15 min at 13,000 rpm before analysis. For CEX-UV analysis, the intact antibody was diluted to 2.0 mg/mL with Mobile Phase A, 10 mM Phosphoric Buffer pH 7.0.

2.3. CE-MS analysis

CE separation was performed on a microfluidic CE platform, ZipChip and coupled to a hybrid quadrupole Orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, Waltham, MA). Native analysis of the intact antibody was carried out with HRN chip with a 22 cm-long separation channel and using the Native BGE provided in the ZipChip Native Antibodies Kit. Native BGE was prepared following the ZipChip protocol. A pre-defined standard native analysis method was used as the starting point. The field strength was adjusted to 500 V/cm and injection volume was 1 nL. Subunit analysis and peptide mapping analysis were carried out with a HR chip with a 22 cm-long of separation channel. A ZipChip Peptide Assay kit was used for peptide mapping analysis. The field strength was adjusted to 400 V/cm and the injection volume was set at 1 nL. The mass spectrometer parameters were 300 °C for the inlet capillary temperature, 2 psi for the sheath gas, 200 V for the S-lens, 50 V for the in-source CID, 17500 for the mass resolution, AGC target at 1×10^6, microscans set to 3, max injection time set to 20 ms. A full scan range from m/z 1500 to 6000 was adjusted for native intact analysis and a range from m/z 800 to 3000 was adjusted for subunit and peptide mapping analysis. Microfluidic CE-MS data was visualized using Thermo Xcalibur QualBrowser (Thermo Fisher Scientific) and deconvolution of the mass spectra was performed using the deconvolution algorithm in Biopharma Finder 2.0 software (Thermo, Fisher Scientific).

2.4. CEX-UV analysis

CEX analysis was performed with a Thermo ProPac WCX-10 column on an Agilent 1260 high-performance liquid chromatography system coupled to a UV detector with a detection wavelength of 280 nm. The mobile phase was a mixture of 10 mM sodium phosphate at pH 7.0 (A) and 10 mM sodium phosphate with 150 mM sodium chloride at pH 6.7 (B). The experiment was performed at ambient temperature, and the total run time was 40 min with a mobile phase flow rate of 0.5 mL/min. The mobile phase gradient was initially held at 10% B for 5 min, increased to 10%–40% B between 5 and 30 min, increased to 100% B at 30.1 min, held at 100% B from 30.1 to 34.9 min, decreased to 10% B at 35 min and finally held at with 10% B for 5min.

3. Results and discussion

In this study, as a complementary method to CEX analysis, a

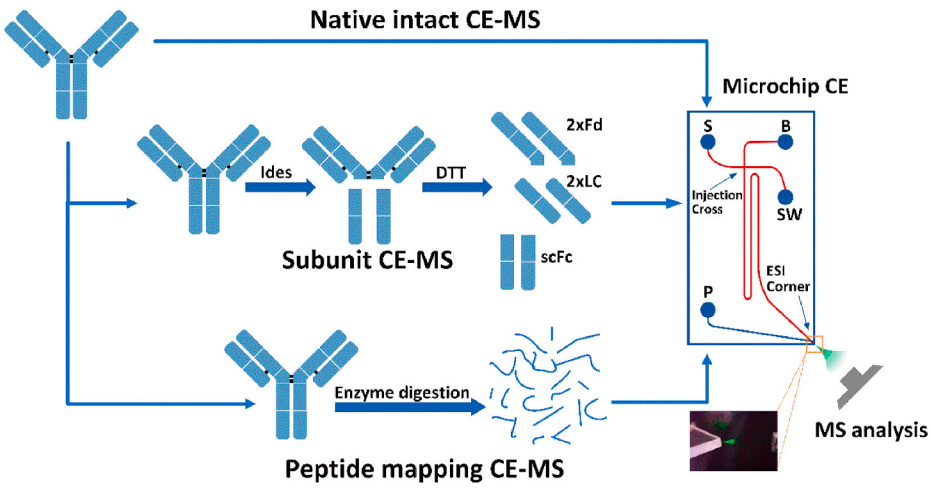


Fig. 1. Workflow of microfluidic capillary electrophoresis–mass spectrometry (CE-MS) for multi-level characterization of antibodies.

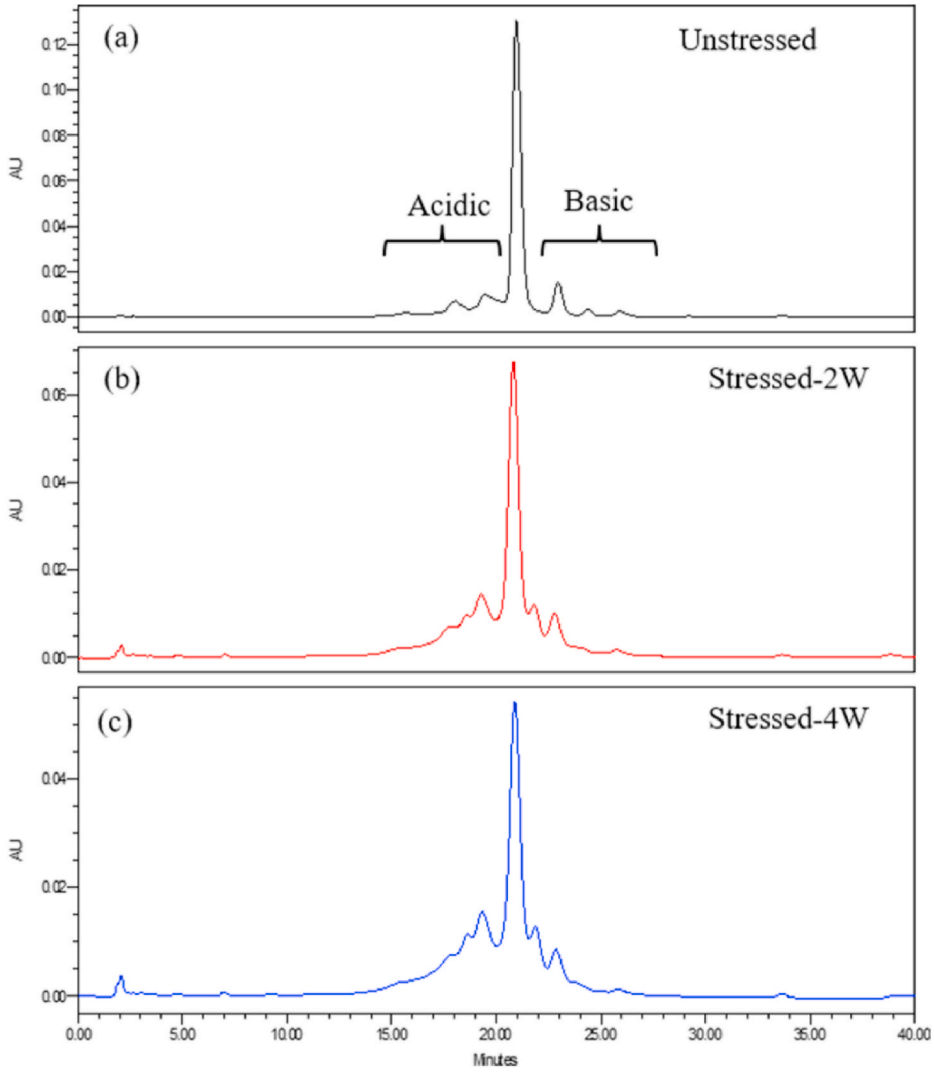


Fig. 2. Cation Exchange Chromatography (CEX) spectra of unstressed mAb and mAbs stressed at 40 °C for 2 weeks(2W) and 4 weeks(4W).

microfluidic CE-MS system was used to elucidate the charge heterogeneity of a mAb. Microfluidic chip-based CE provides multi-level analysis of antibodies, including intact, subunit, and digested peptides (Fig. 1). In addition, the straightforward "dilute and shoot" workflow does not involve complicated sample preparation and enables monitoring of the degradation pathways of stressed antibodies at different time points.

CEX is a common method that can be used for separation of charge variants of unstressed and stressed antibodies (Fig. 2). Fig. 2 (a) shows

Table 1
Changes in the peak areas in CEX with increased stress time.

Sample Name	Area%		
	Acidic Peak	Main Peak	Basic Peak
Unstressed	20.7	66.7	12.6
Stressed-2W	27.5	51.2	22.3
Stressed-4W	35.7	39.9	25.0

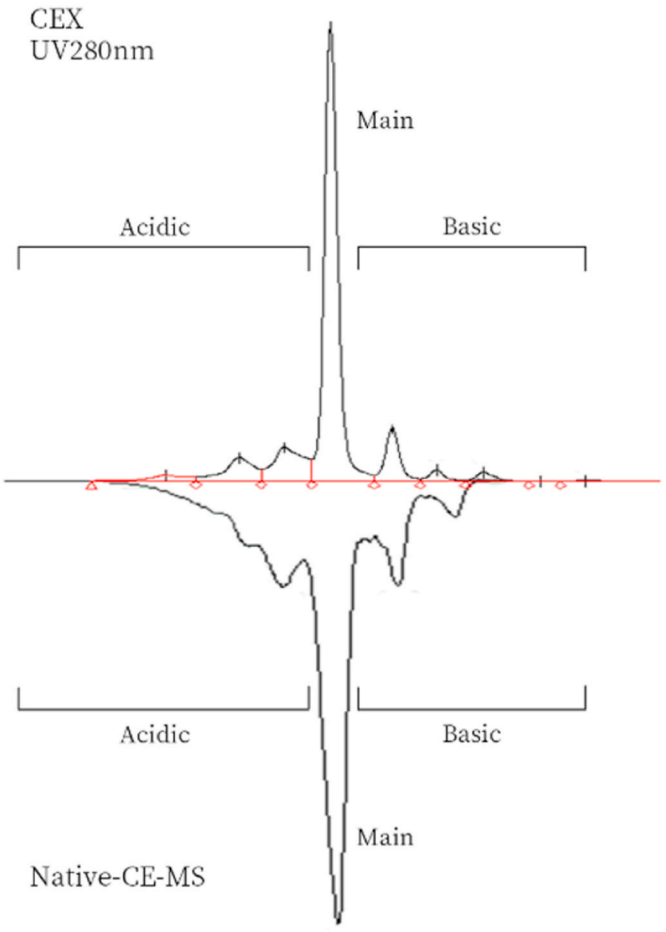


Fig. 3. Comparison of the separation of mAb using CEX (top spectrum) and Native-CE-MS (bottom spectrum).

the spectrum of unstressed sample charge variants (Unstressed). The charge variants of samples stressed for 2 weeks (Stressed-2W) and 4 weeks (Stressed-4W) are shown in Fig. 2(b) and (c). The charge variant peak profiles changed with time and the peak area percentages for all variants for the Unstressed, Stressed-2W, and Stressed-4W samples are summarized in Table 1. As the time the sample was stressed for increased, the percentages of the acidic and basic peak areas increased from 20.7% to 35.7% and from 12.6% to 25.0%, respectively. The change in the acidic and basic peaks were closely related to the mAb structure. Further details on the mass and structure for each variant were not available from the CEX analysis.

Microfluidic CE-MS of antibodies in their native state (Native-CE-MS) is straightforward and maintains the native structure of the antibody. The Native-CE-MS spectrum of the unstressed antibody is shown in Fig. 3. The acidic and basic peaks were defined according to their retention/migration time relative to the main peak. For example, in CEX the acidic peaks eluted before the main peak, whereas in Native-CE-MS, the acidic peaks eluted after the main peak. For ease of comparison, the spectra obtained by of CEX in (Fig. 2[a]) and Native-CE-MS are aligned in Fig. 3 with the Native-CE-MS spectrum processed and rotated by 180°. The results clearly demonstrated that the separation of acidic and basic peaks was consistent with the two methods. Therefore, Native-CE-MS provides complementary support to CEX analysis. The additional MS information obtained by Native-CE-MS will allow for in-depth analysis of mAbs at the intact, subunits, and peptides levels. Stressed samples were monitored to further understand the degradation pathways of the mAb. Samples are separated in CEX according to the elution properties of the protein and chromatographic column. Separation by CE, which is typically capillary zone electrophoresis, is based on the differences in the net charges and hydrodynamic radii of the components to be separated under the action of a neutralizing electric field in an electrolyte solution. CEX uses a UV detector, whereas CE is hybridized with MS, and different detector types can produce different signal responses. Therefore, some differences are expected between the two methods for the peak positions of acidic and basic variants.

3.1. *Unstressed sample*

The mAb was analyzed by Native-CE-MS. Two basic Peaks (B1 and B2), a main peak (M), and two acidic Peaks (A1 and A2) were clearly distinguished (Fig. 4a). The peaks were deconvoluted and the deconvoluted spectra are shown in Fig. 4b–f, and the deconvoluted masses are summarized in Table 2. The molecular weights of peaks M, B1, B2, A1, and A2 were determined by comparison with the theoretical molecular weights. The detected molecular weights for peak M were 147,732.41 Da and 147,893.58 Da, and these matched well with the theoretical molecular weights of 147,732.85 Da and 147,895.01 Da, respectively (Table 2). The molecular weight of 147,732.41 Da was attributed to two heavy-chain N-terminal pyroglutamic and C-terminal lysine clipping, G0F/G0F glycosylation profiles (2Gln-›Pyro-Glu/-2Lys Loss/G0F/G0F). The molecular weight of 147,893.58 Da was attributed to two heavy chain N-terminal pyroglutamic and C-terminal lysine clipping, G0F/G1F glycosylation profiles (2Gln-›Pyro-Glu/-2Lys Loss/G0F/G1F). For basic variants, the molecular weight of peak B1 was147,674.98, which was 58 Da smaller than the molecular weight of peak M. The molecular weight of peak B2 was 17,619.06, which was 114 Da smaller than the molecular weight of peak M. According to the literature [12,20], B1 is formed by C-terminal amidation in one chain (2Gln-›Pyro-Glu/-2Lys Loss/-Gly loss/Pro + amide/G0F/G0F) and B2 is formed by C-terminal amidation in two chains (2Gln-›Pyro-Glu/-2Lys Loss/-2Gly loss/2Pro + amide/G0F/G0F). For acidic variants, the molecular weight of peak A1 was 147,735.06 Da, which is 2 Da larger than peak M. The molecular weight of peak A2 was 147,743.84 Da, which was11 Da larger than the molecular weight of peak M. As reported in the literature [6], deamidation is a major contributor to formation of acidic variants. The difference between A1 and the main peak was 2 Da, and that between A2 and the main peak was 11 Da. Deamidation would produce a change of 1 Da, and the larger increases in the molecular weight that we observed may have been caused by modification at multiple sites or multiple times at the same site. We concluded that A1 and A2 were products of deamidation. The two peaks in the figure could be attributed to different glycan modifications.

The ScFc, light chain, and Fd subunits of mAb were then separated analyzed by microfluidic CE-MS. (Fig. 5a). The intact mAb samples were first treated with IdeS and then DTT was added to reduce the protein and generate the three subunits (ScFc, light chain, and Fd). IdeS cleaves mAb in the lower hinge region between two glycans, creating a F(ab')2 and two single chain ScFc domains [21,22]. During CE separation of these three subunits, the ScFc subunit migrates first, followed by the light chain subunits, and then the Fd subunit.

The deconvoluted spectra of the ScFc, light chain, and Fd subunits are shown in Fig. 5b, c, and 5d, respectively, and the deconvoluted molecular weights are summarized in Table 3. The assignment of each peak was confirmed by comparison with the theoretical molecular weight. The detected molecular weights for the ScFc subunit were

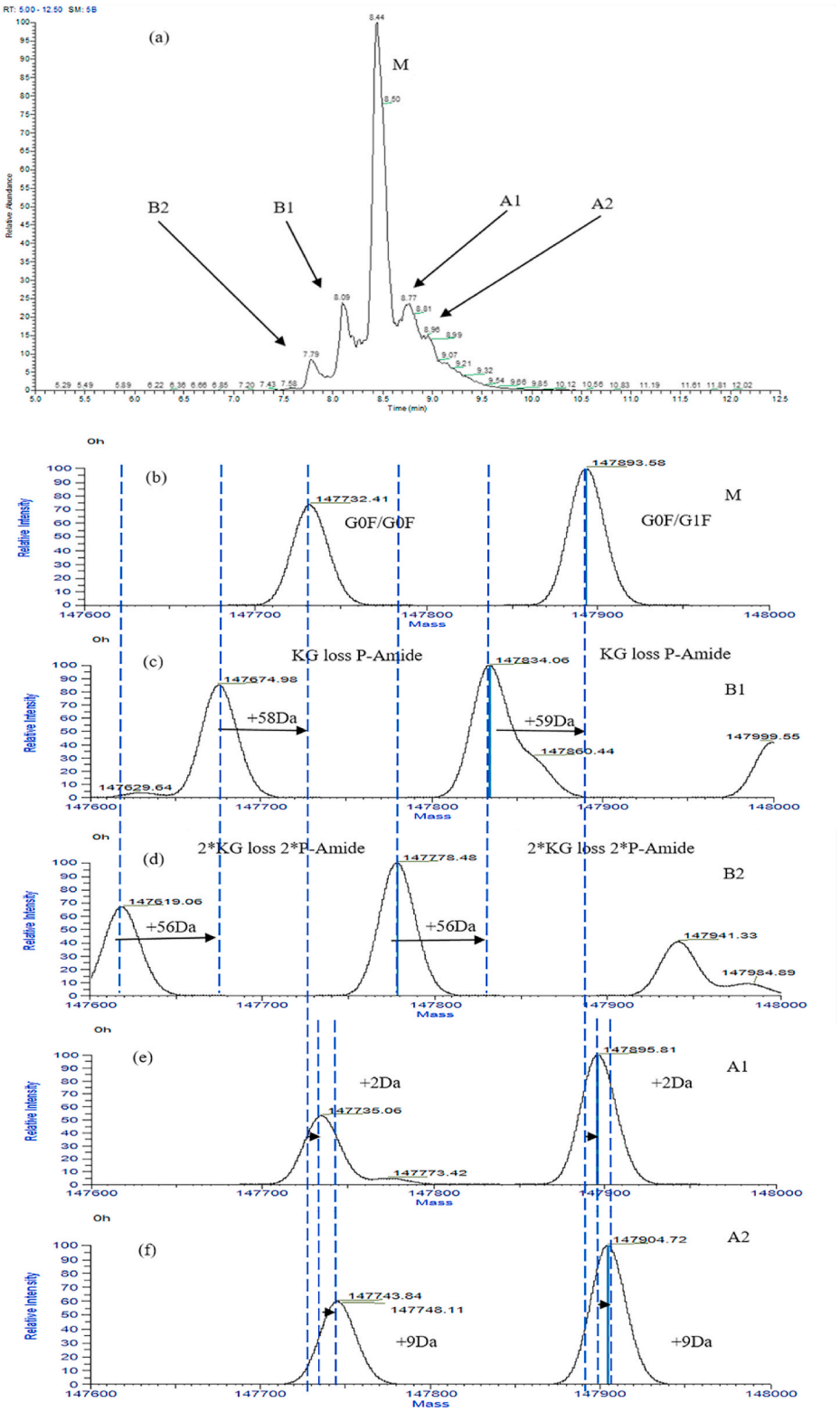


Fig. 4. CE-MS separation of charge variants of unstressed mAb and the deconvolution results for basic and acidic variants. (a)Total ion electropherograms (b) Main Peak deconvoluted spectra (c) Basic Peak 1 deconvoluted spectra (d) Basic Peak 2 deconvoluted spectra (e) Acidic Peak 1 deconvoluted spectra (f) Acidic Peak 2 deconvoluted spectra.

25,199.32 Da, 25,361.34 Da and 25,523.46 Da, and these matched well with the theoretical molecular weights of 25,200.01 Da, 25,362.17 Da and 25,524.27 Da, respectively (Table 3). The molecular weight of 25,199.32 Da was attributed to C-terminal lysine clipping and G0F glycosylation profiles (ScFc -Lys Loss/G0F), the molecular weight of 25,361.34 Da was attributed to C-terminal lysine clipping and G1F glycosylation profiles (ScFc -Lys Loss/G1F), and the molecular weight of 25,523.46 Da was attributed to C-terminal lysine clipping and G2F glycosylation profiles (ScFc -Lys Loss/G2F). The detected molecular weight for the light chain and Fd (Gln-)Pyro-Glu) subunits were

Table 2
Summary of the theoretical molecular weight and measured molecular weights at the intact proteins level.

Peak ID	Modification	Theoretical Mass (Da)	Measured Mass (Da)	Error (Da)
M	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/G0F/G0F	147732.85	147732.41	−0.44
	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/G0F/G1F	147895.01	147893.58	−1.43
B1	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/-Gly Loss/Pro + amideG0F/G0F	147674.85	147674.98	0.13
	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/-Gly Loss/Pro + amideG0F/G1F	147837.01	147834.06	−2.95
B2	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/-2Gly Loss/2Pro + amideG0F/G0F	147616.84	147619.06	2.22
	Intact:2Gln-⟩Pyro-Glu/-2Lys Loss/-2Gly Loss/2Pro + amideG0F/G1F	147779	147778.48	−0.52

23,954.90 Da and 24,732.05 Da respectively, and these matched well with the theoretical molecular weights of 23,955.67 Da and 24,732.79 Da.

3.2. Stressed samples

Native-CE-MS was used to analyze the stressed samples. Unstressed mAb and mAb stressed for 2 weeks and 4 weeks were characterized and the spectra are shown in Fig. 6. The deconvoluted spectra for M, B1, B2, A1, and A2 for the unstressed and stressed mAbs are shown in Fig. 6 (a) and Figs. S1–S4. The results clearly demonstrated an increase in mass from the unstressed to stressed samples for all variants, and an increase

Table 3
Summary of the theoretical molecular weight and measured molecular weight of protein subunits.

Modification	Theoretical Mass(Da)	Measured Mass(Da)	Error (Da)
ScFc -Lys Loss/G0F	25200.01	25199.32	−0.69
ScFc -Lys Loss/G1F	25362.17	25361.34	−0.83
ScFc -Lys Loss/G2F	25524.27	25523.46	−0.81
LC	23955.67	23954.90	−0.77
Fd Gln-⟩Pyro-Glu	24732.79	24732.05	−0.74

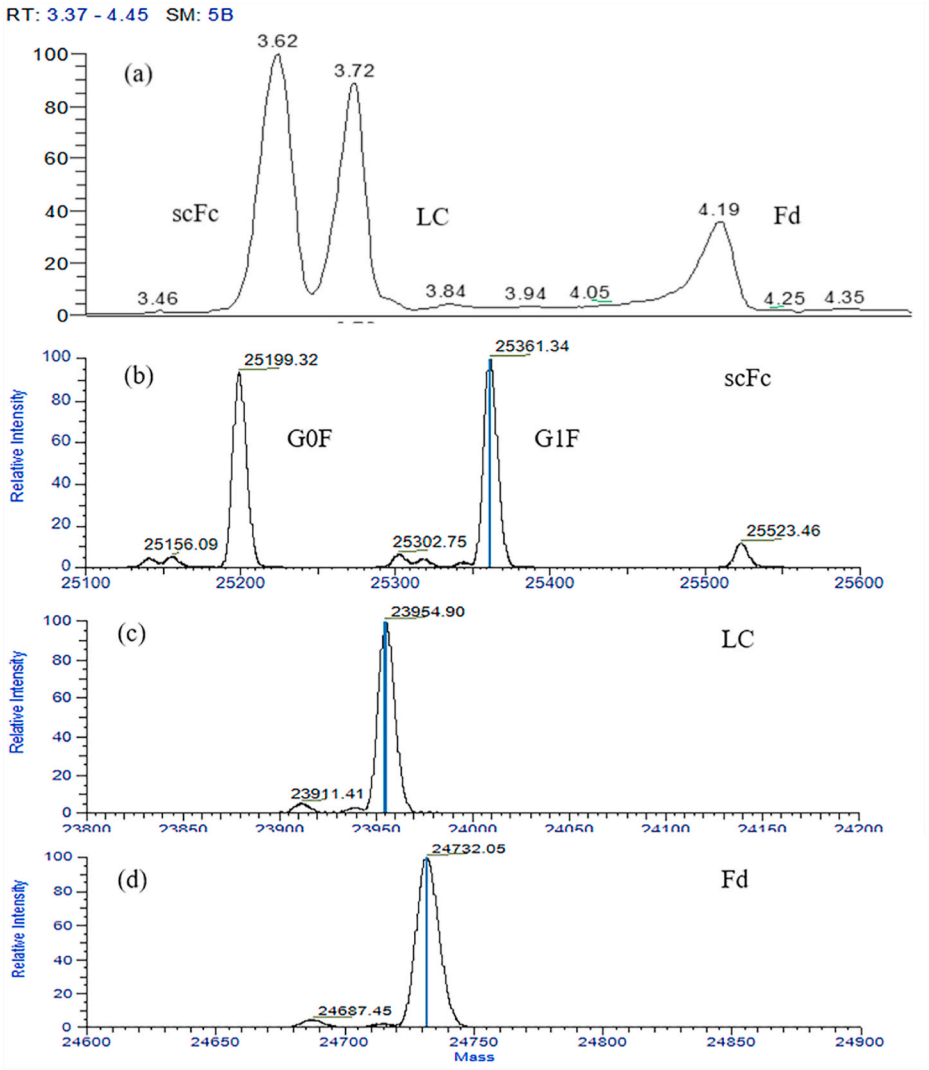


Fig. 5. Total ion electropherogram of mAb subunits and deconvolution results for the ScFc subunit, light chain and Fd subunit. (a)Total Ion electropherograms (b) scFc deconvoluted spectra (c) light chain (LC) deconvoluted spectra (d) Fd deconvoluted spectra.

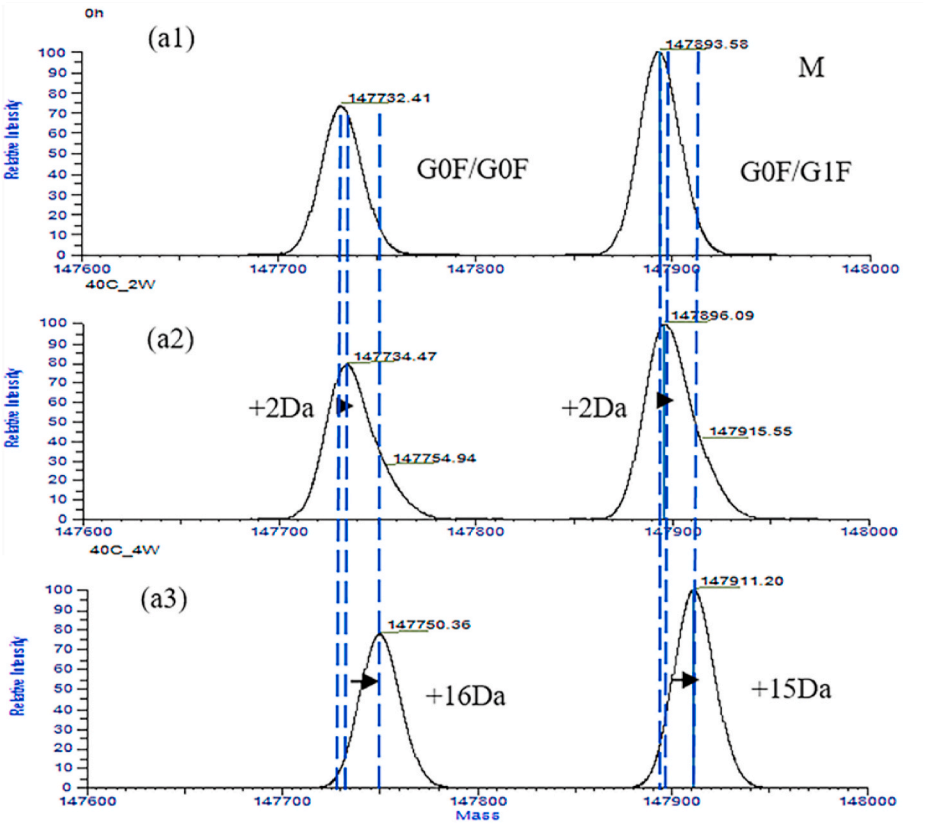


Fig. 6. Comparison of the deconvoluted intact mass of charge variants of stressed mAb (a1) The unstressed sample (a2) the sample stressed for 2 weeks (a3) the sample stressed for 4 weeks.

Table 4
Native intact mass analysis for monitoring of stressed samples.

Peak	G0F/G0F		
	Unstressed	Stressed-2W	Stressed-4W
M	147732.41	147734.47	147750.36
A1	147735.06	147740.06	147754.48
A2	147743.64	147746.75	147756.56
B1	147674.98	147721.14	147734.00
B2	147619.06	147683.36	147700.30

in mass along with the stress time. The spectra showed mass shifts for M between the stressed samples and unstressed samples of 2 Da for the 2-week stressed sample and 16 Da for the 4-week stressed sample. The mass of G0F/G0F was compared for all variants (Table 4). Mass shifts of 5 Da for A1 and 3 Da for A2 were observed between the 2-week stressed sample and the unstressed sample. Mass shifts of 15 Da for A1 and 10 Da for A2 were observed between the 4-week stressed sample and 2-week stressed sample. Mass shifts of 47 Da for B1 and 64 Da for B2 were observed between the 2-week stressed sample and the unstressed sample, and mass shifts of 13 Da for B1 and 17 Da for B2 were observed between the 4-week stressed sample and 2-week stressed sample. From the results for the main peak and acidic peaks, we inferred that the most likely modification causing the mass shifts was deamidation, which is consistent with other reports in the literature [3]. The large mass shifts observed for the basic peaks in the spectra seem unlikely to arise from deamidation alone. This suggests that there may be additional structural modifications. According to the mass shift values, the modifications probably involve both deamidation and oxidation. Apart from the G0F/G0F glycoform, all glycoforms showed the same trend for the mass shift. To verify the structural modification occurring with the antibody,

Table 5
Subunit mass analysis for monitoring of stressed samples.

Peak	G0F/G0F		
	Unstressed	Stressed-2W	Stressed-4W
LC	23954.90	23955.01	23955.05
ScFc	25199.32	25216.61	25215.49
Fd	24732.05	24750.02	24749.07

analyses were performed at the subunit and peptide levels (see Table 5).

The subunit of stressed and unstressed antibodies were analyzed by CE-MS and the deconvoluted spectra are shown in Fig. 7. The subunits masses greatly increased with increases in the stress time and a mass shift of 16 Da was observed for both ScFc and Fd subunits. This mass shift may be caused directly by oxidation. For the light chain (LC) subunit, the mass did not change with increases in the stress time, which suggested that oxidation did not occur in the light chain. In the heavy chain (HC), oxidation occurred in both the ScFc and Fd subunits, and this was consistent with the above hypothesis that the change in the basic peak was caused by modifications at multiple sites. A mass shift of 17 Da in the Fd subunit was observed for all three samples (unstressed and stressed), and this clearly indicated that oxidation occurred. Meanwhile, the peak intensity of variant 1 increased with time, which was consistent with the results from intact-level analysis. This could be explained by digestion of the subunit in a denatured condition, which would change the protein structure to a stretched configuration unlike the globular structure under native conditions. Consequently, it would not be possible to observe the separation of charge variants at the subunit level.

Peptide mapping of the stressed samples was performed and the CE spectrum is shown in Fig. 8. According to the theoretical sequence, the

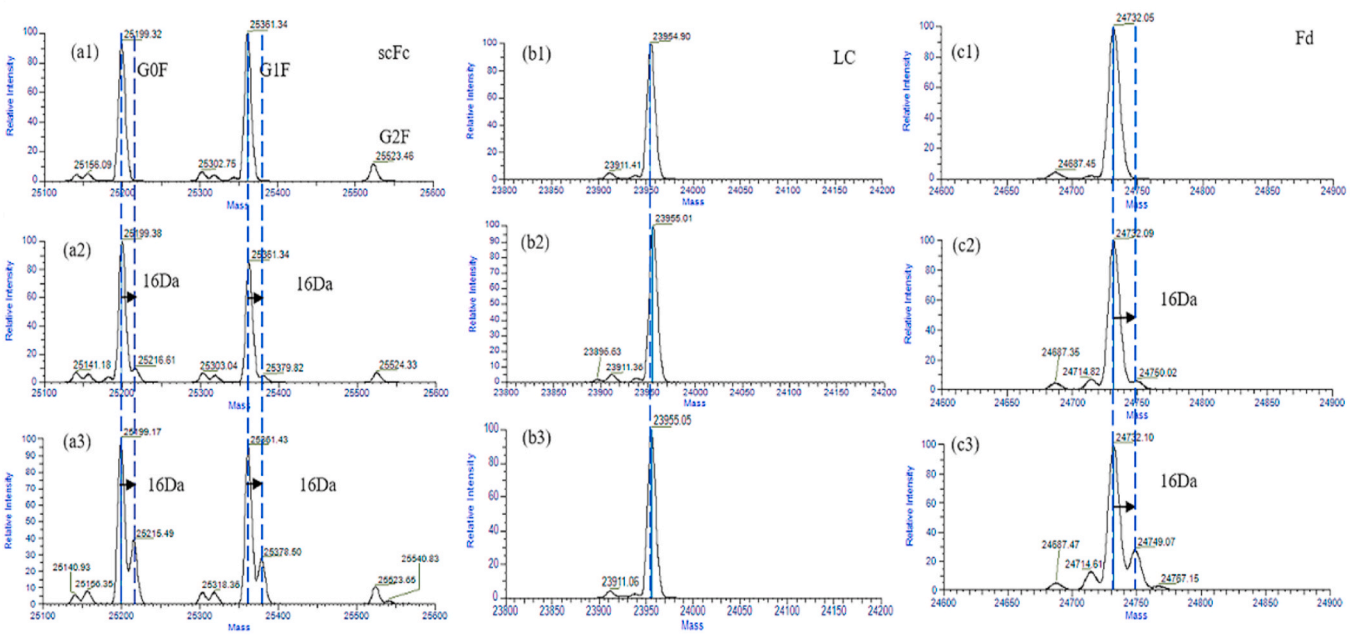


Fig. 7. Deconvoluted intact mass of mAb subunits: (a) LC subunit; (b)ScFc subunit; (c) Fd subunit; (1) The unstressed sample (2) the sample stressed for 2 weeks (3) the sample stressed for 4 weeks.

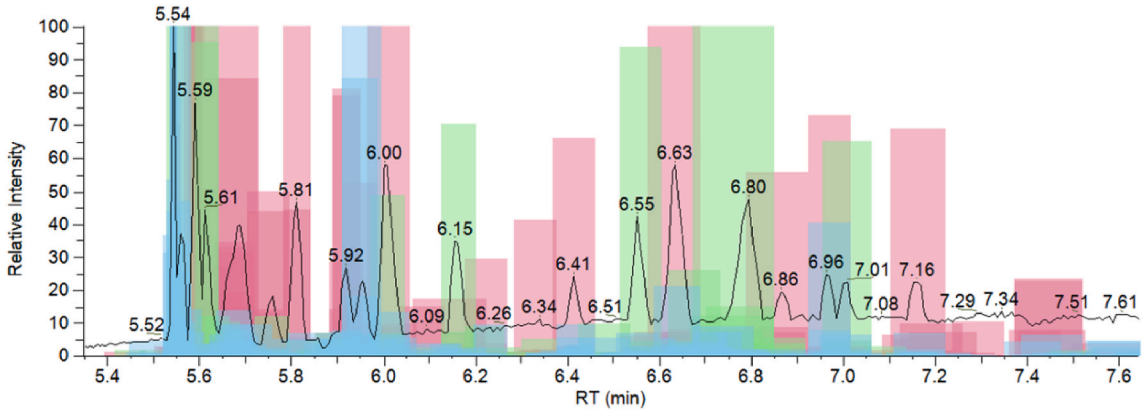


Fig. 8. Total ion electropherogram of tryptic peptides of mAb. Data visualization was performed using Thermo BioPharma Finder 2.0. Colors: pink, peptide heavy chain; green, peptide light chain; and light blue, unidentified peptide. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 6
Peptide level analysis of significant post-translational modification (PTM) changes in stressed samples.

Protein	Modification	%Abundance Unstressed	%Abundance Stressed-2W	%Abundance Stressed-4W
HC	M48+Oxidation	15.9	15.2	18.5
HC	W50+Oxidation	0.0	2.8	6.8
HC	M109+Oxidation	4.1	12.1	29.7
HC	M249+Oxidation	4.0	14.6	31.9
HC	N283+deamidation	0.9	1.0	1.4
HC	N381+deamidation	14.0	14.8	15.5

data for the unstressed and stressed (2 week and 4 week) samples were analyzed using Thermo BioPharma Finder 2.0. The results showed significant differences in post translational modifications (PTMs) (Table 6) for the heavy chain M 48(15.9%–18.5%), W50 (0.0%–6.8%), M109 (4.1%–29.7%), and M249 (4.0%–31.9%) sites with oxidation, the heavy chain N283 (0.9%–1.4%) and N381 (14.0%–15.5%) sites with deamidation The peptide-level results further confirmed the oxidation and deamidation occurred at multiple sites in the heavy chain, and that the heavy chain mass increased for stressed samples, The results were complementary to those at the intact and subunit levels.

The monitoring results for stressed samples from Native-CE-MS, and subunit and peptide mapping confirmed the hypothesis that acidic variants and main variants in stressed sample were formed by deamidation, while basic variants in stressed samples were formed by deamidation and oxidation. Native-CE-MS is a straightforward method that can be used to monitor changes and their trends for antibodies under stress. Unlike for intact-level analysis, it is difficult to observe deamidation of antibodies in subunit-level analysis. However, it is a good way to identify certain types of modifications, especially oxidation. Analysis at the subunit and peptide levels with a reliable method, provide an efficient means for evaluation of the degradation pathway(s) of mAbs. Native-CE-MS offers several benefits, such as fast analysis and minimal sample preparation, when combined with analytical separation in advance, which will avoid artificially induced modifications of the

analyte.

4. Conclusion

CEX can be used to monitor the charge heterogeneity of mAbs. CE-MS can provide a basis for the identification of chromatographic peaks in CEX spectra. Charge variants of a recombinant mAb were thoroughly characterized by CE-MS of the native mAb and its subunits. CEX-UV is widely used in industry at present because it provides stable quantitation, and it is simple and inexpensive; however, there are issues with characterization of separated peaks. MS is a well-known powerful analytical method for elucidation of chemical and biological compounds, and CE-MS is recommended as a characterization method to monitor antibodies. Our results demonstrate that basic variants of recombinant mAb are formed by C-terminal amidation, while acidic variants are formed mostly by deamidation. The degradation pathways of samples under temperature stress were monitored at the intact protein, subunit, and peptide level. The results demonstrate that the degradation pathway for the main peak and acidic peak involve deamidation, and the degradation pathway for basic peaks involves oxidation and deamidation. The characterization of charge variants is a powerful strategy for determining post-translational modifications and their impact on chromatographic behaviors.

Credit author statement

Qiang Sun: Conceptualization, Methodology, Formal analysis, Writing - Original Draft. **Liang Wang**: Data Curation, Writing - Review & Editing. **Na Li**: Resources, Formal analysis. **Liming Shi**: Supervision, Project administration.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work “Characterization and monitoring of charge variants of a recombinant monoclonal antibody using microfluidic capillary electrophoresis-mass spectrometry (CE-MS)”, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in or the review of the manuscript entitled.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ab.2021.114214.

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