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Investigation of N-terminal glutamate cyclization of recombinant monoclonal antibody in formulation development

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Abstract

The N-terminal glutamic acid (Glu) can be cyclized to form pyroglutamate (pGlu). Recent studies have suggested that N-terminal pGlu formation is an important posttranslational or co-translational event and is greatly facilitated by the enzyme glutaminyl cyclase, although the impact of the N-terminal cyclization on the potency and overall stability of mAbs is not been well known. Since most recombinant monoclonal antibodies (mAbs) contain glutamic acid and/or glutamine at their N-terminus, understanding the cyclization mechanisms may shed light on the factors that control the pGlu formation in therapeutic mAb development.

Here, two mass spectrometry-based techniques were developed to investigate *N*-pyroglutamyl formation and the high conversion rate to pGlu at the N-terminus of the mAb was reported in the formulation development. The pGlu formation is favored at pH 4 and 8, but is less common at the neutral pH that is optimum for the enzymatic Glu conversion. These observations suggest that pGlu formation can proceed non-enzymatically at mild conditions and that this cyclization is not driven by glutaminyl cyclase in non-physiological conditions. We also calculate the half-lives of the N-terminal Glu at different pH and temperatures from the kinetics data, which would be very helpful for predicting pGlu formation and for selecting proper formulation and storage conditions.

Keywords: Cyclization; *N*-Pyroglutamic acid; Glutamic acid; N-terminal; Monoclonal antibody; Peptide mapping; Liquid chromatography/mass spectrometry

1. Introduction

With the mastery of a robust cell line, large scale cell culture technology and highly efficient purification process, more and more recombinant monoclonal antibodies (mAbs) have been developed to fight diseases in many therapeutic areas [1,2]. Like other recombinant proteins, therapeutic mAbs are subject to a variety of chemical modifications that may occur during protein expression, purification, transportation and storage. These modifications include but are not limited to oxidation, deamidation, proteolysis cleavage, disulfide-bond scrambling, glycosylation and cyclization. These reactions, enzymatic or non-enzymatic, could affect the size or charge heterogeneity of mAbs, and may modify their antigen binding affinity. The impact of these modifications on the overall stability of the mAbs, their bioactivities, and therapeutic potency depends not only on just the modifications themselves but also on their locations [3–10]. Therefore, therapeutic mAbs also require extensive and stringent characterization of purity, structural integrity and stability. It is of a great interest to investigate the factors that affect these chemical modifications during therapeutic drug development.

It has been well known that the amino terminus of mAbs can be modified through acetylation, formylation and pyroglutamylation. Among them, pyroglutamate (pGlu) formation is of special interest because most mAbs have glutamine (Gln) or glutamic acid (Glu) at the amino terminus. Previous studies have shown that pGlu was almost exclusively formed in vivo by intramolecular Gln cyclization [11]. It was believed that the pGlu formation was an enzymatic reaction because Wilson and Cannan reported that the conversion of Glu to pGlu could only occur at relatively harsh incubation at 100 °C for 50 h [12,13]. The reaction is even slower in solutions of weak acids or weak bases. At strong acid or alkaline conditions the conversion of Glu to pGlu is rapid and practically complete. Other studies have reported that N-terminal pGlu can be derived from Glu without prior conversion to Gln and indicated that

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N-terminal pGlu formation from Glu must be an enzymatic reaction rather than a spontaneous chemical process [14]. The spontaneous cyclization of an N-terminal glutamine occurs only slowly under physiological conditions [15]. Recent studies have suggested that N-terminal pGlu formation is an important post-translational or co-translational event and is greatly facilitated by the enzyme glutaminyl cyclase [16,17]. If the above findings proved that the mechanism of N-terminal pGlu formation is an enzymatic reaction under physiological conditions, a non-enzymatic mechanism should be examined, which may occur in non-physiological conditions.

In this report, we have investigated the pGlu formation from Glu at the N-terminus of a mAb under weak acids or bases condition during therapeutic drug development. The mAb is different from other typical mAbs containing N-terminal Glu and its N-terminal Glu can be converted to pGlu non-enzymatically. The cyclization reaction is chemical process for the mAb during the formulation development. In this study, the two mass spectrometric-based methods were used to facilitate the investigation of the pGlu formation at the N-terminus of the mAb. The first method, a bottom-up method, is a proteolytic approach with trypsin digestion to identify the N-terminal pGlu by HPLC with electrospray ionization/ion trap mass spectrometry (ESI-MS trap). The second one uses high resolution ESI-QTOF MS to analyze the formation of pGlu at the N-terminus of the same mAb that is reduced but not further digested by protease to minimize sample treatment induced pGlu formation (a top-down method). The two methods gave comparable results: high percentage pGlu was observed for the studied mAbs of our formulated sample under high temperature shelf storage. These findings demonstrated that pGlu formation could occur under storage condition without facilitating by the enzyme.

2. Experimental

2.1. Materials

Recombinant mAb was produced and purified at Amgen Inc. The purified mAb was in 10 mM sodium acetate buffer with pH 5.2 and stored at −80 °C. Trypsin of sequencing grade was purchased from Promega and stored at −20 °C. All chemicals and reagents were of analytical grade and purchased from Sigma. Other formulated samples were prepared through dialyzing into the final formulation solutions using 10 MWCO Snakeskin dialysis bags.

2.2. Reduction, alkylation and tryptic digestion

Approximately 1 mg of the mAb was denatured with 6 M guanidine HCl, 0.25 M Tris–HCl, 1 mM EDTA, at pH 7.5. Ten microliters of 0.5 M dithiothreitol (DTT) was added to the solution and the reaction mixture was placed at 37 °C for 30 min. After the sample was cooled to room temperature, 24 μl of 0.5 M iodoacetamide (IAA) was added and the sample was incubated at room temperature in the dark for 30 min. Ten microliters of 0.5 M DTT was added to the samples in order to terminate the alkylation reaction. Approximately 500 μl of reduced and alkylated material was buffer exchanged with 950 μl of 0.1 M Tris–HCl to a final concentration of 1 mg/ml of antibody, using a NAP-5 column (Amersham BioSciences, Uppsala, Sweden) equilibrated with 10 ml of 50 mM Tris, 1 mM $CaCl_2$, pH 7.0. Tryptic digestion was performed for 5 h at 37 °C using an enzyme:protein ratio of 1:55. The digests were then refrigerated at 4 °C for analysis.

2.3. HPLC separation of tryptic peptides

The tryptic peptides were separated by reversed-phase HPLC using an Agilent 1100 HPLC equipped with a diode-array detector, autosampler, flow cell and temperature controlled column compartment (Agilent, Palo Alto, CA, USA). A Varian Metachem Polaris C18 column (150 mm × 2.1 mm i.d.) packed with a 3 μm nominal diameter, 300 Å pore size C_{18} resin (Varian, Torrance, CA, USA) was used for the separations. The solvents were—A: 0.1% trifluoroacetic acid (TFA; Pierce, Rockford, IL, USA) in water, and B: 0.1% TFA in 90% acetonitrile (Baker, Phillipsburg, NJ, USA). The column was equilibrated at 0% solvent B. The two-stage gradient was from 0 to 17% B within 17 min, then followed by a second gradient from 17 to 38% B within 90 min. A flush step was performed with 90% B for 10 min and the column was equilibrated with 0% B for 10 min. The column temperature was maintained at 40 °C. The absorbance of the eluent was monitored at 214 nm.

2.4. Mass spectrometry analysis of tryptic peptides

The HPLC was directly coupled to Agilent MSD ion trap mass spectrometer (Agilent, Palo Alto, CA, USA) equipped with an electrospray ionization source. The spray voltage was 4.5 kV and the capillary temperature was 350 °C. The fragmentation mass spectra were obtained using ion trap collision energies of 35%. Each full scan mass spectrum was followed by a zoom scan, followed by a data dependent MS/MS scan of the most intense peak. The Dynamic Exclusion feature was enabled (repeat counts, 2; repeat duration, 0.3 min; exclusion duration, 5 min and exclusion mass width, 2 Da).

2.5. HPLC separation of reduced antibodies

The reduced mAb was analyzed using an Agilent 1100 HPLC unit equipped with a diode-array detector, autosampler, micro flow cell and temperature controlled column compartment (Agilent, Palo Alto, CA, USA). Column was heated at 35 °C to enhance separation. Mobile phase A consisted of 0.1% formic acid in water and mobile phase B consisted of 80% *N*-propanol, 10% acetonitrile, 10% H_2O and 0.1% formic acid. Separation was performed on a Zorbax SB CN 150 mm × 1.0 mm 3.5 μm 300 Å (50 μl/min and 4 μg injection). The column was equilibrated at 10% solvent B. One minute after sample injection the concentration of buffer B was increase to 28% over 1 min followed by a linear gradient of 28% to 35% B over 33 min. The column was re-equilibrated by ramping up buffer B to 100% over

1 min, 5 min at 100% B, dropping down to 10% B over 1 min followed by 5 min at 10% B. UV absorption was monitored at 214 nm.

2.6. Mass spectrometry analysis of the reduced antibodies

Mass spectrometry was performed on a Micromass QTOF *Micro* mass spectrometer through an electrospray ionization (ESI) atmosphere–vacuum interface. The ESI-QTOF mass spectrometer was set to run in positive ion mode with a capillary voltage of 3400 V, sample cone voltage of 40 V, an *m/z* range of 1000–5500, with a mass resolution of 5000. The instrument was tuned and calibrated using multiply charged ions of antibody. The deconvolution of electrospray ionization mass spectra was performed using a MaxEnt1 algorithm, which is a part of the MassLynx software from Micromass.

2.7. Fourier transform infrared (FT-IR) spectroscopy

The cyclized mAb was analyzed by an ABB Bomem (Quebec, Canada) MB-series FT-IR spectrometer. The sample was prepared and infrared spectra were obtained according to published procedures [18]. The protein solution was loaded into a BioCell (Bio Tools) with CaF2 windows having a 6.5-μm fixed path length well. For each spectrum, a 128-scan interferogram was collected in a single-beam mode with a 4 cm^{-1} resolution. The reference spectrum was recorded under identical scan conditions. Second-derivative spectra were obtained with a seven-point Savitsky–Golay derivative function. Final spectra were treated with a 2× fast Fourier transform (FFT) interpolate function and plotted with SigmaPlot 8.0 software (Systat Software).

3. Results and discussion

The studied mAb contains Glu at N-terminus of both light chain (LC) and heavy chain (HC). The corresponding predicted peptides are EIVLTQSPGTLSLSPGER (LC) and EVQLVESGGGLVQPGGSLR (HC). N-terminal cyclization of Glu was observed at both LC and HC of the studied mAb, which was formulated at 30 mg/ml and stressed at pH 4.0–8.0 and several temperatures up to 45 °C for up to 3 months.

3.1. N-Pyroglutamate identification and confirmation

Tryptic digestion followed by HPLC separation and mass spectrometry analysis of peptides resulted in identification of *N*-pyroglutamyl formation. Fig. 1A gives the total ion chromatogram (TIC) of tryptic mapping. The mass spectra of the native N-terminal peptide peak and its modified peptide peak were shown Fig. 1B and C. The ion at *m/z* 942.7 is the doubly charged mass for the native tryptic peptide in Fig. 1B and the ion at *m/z* 933.8 corresponds to the cyclized tryptic peptide in Fig. 1C. Two doubly charged masses have a change of 9 Da in mass, indicating an 18 Da loss due to cyclization from Glu to pGlu. The cyclization occurred at the N-terminus of the LC and HC of the mAb. To verify these findings, tandem mass spectrometry analysis was performed to confirm the peptide containing pGlu on the LC and HC. Fig. 2 shows the MS/MS spectra of the four peptides, the native peptides and its pGlu containing peptides of the LC and HC. Fig. 2A and B show the tandem mass spectrum and the b, and y ion assignments for the native peptides of the LC and HC, which match the predicted masses. For pGlu containing peptides, Fig. 2C and D show that all y ions have the same mass as that of the native peptide, but all b ions lost 18 Da, resulting in the verification of pGlu and modification occurred at the N-terminus of the LC and HC.

Analyses also showed that the starting material of the studied mAb, purified bulk, did not contain detectable pGlu. Measurements have been taken to minimize sample handling induced pGlu formulation, such as the top-down method using high resolution ESI-QTOF MS with limited sample treatment. Analysis of control samples demonstrated that sample treatment, including tryptic mapping, did not contribute to pGlu formation at the N-terminus of LC. These experimental results showed that the N-terminal cyclization of LC and HC occurred in shelf storage, a non-physiological condition.

A top-down method was employed to demonstrate the formation of pGlu at the terminus of light LC. In Fig. 3, deconvoluted mass spectrum gives the mass 23,579 Da in the native LC peak and the mass 23,561 Da in the pGlu containing LC peak with 18 Da due to cyclization of the antibody. Data generated from the sample at different pH and buffers were analyzed by the bottom-up and top-down methods. In the peptide mapping method, the percentage of pGlu was determined from the integration of the extracted ion chromatogram (XIC) of the native peptide peak mass and pGlu peptide peak mass. The top-down method data was based upon the integration of two deconvoluted mass peak area of the native LC from the purified bulk and modified LC from the stressed samples. Table 1 listed the results from 3-month storage samples at different pH and buffers, showing a good correlation on the pGlu % of these mAb samples.

3.2. Effects of formulation conditions on N-pyroglutamyl formation and its rate

Temperature, pH and storage time were investigated on their impact on pGlu formation of the studied mAb. The pGlu formation at the N-terminus of the LC and HC increased with elevated temperature and lowered pH over 3 months (Fig. 4A and B). The pGlu formation of the HC was obviously slower than that of the LC no matter what pH, temperature and storage time levels. We could not detect any N-terminal pGlu formation on HC when temperature was at 29 °C or lower, even after 3-month storage. At the N-terminus, a significant amount of pGlu was observed on the LC when stored at 37 and 45 °C for 1 month, much faster than the HC. The mechanism that led to the difference is still to be explored. The extended pH effect data was collected from 3 months at 37 and 45 °C (Fig. 5). Within the pH range of 4–6, high amount of pGlu was generated at pH 4 and the minimal pGlu was formed at pH 6. Within the pH range of 6–8, the pGlu

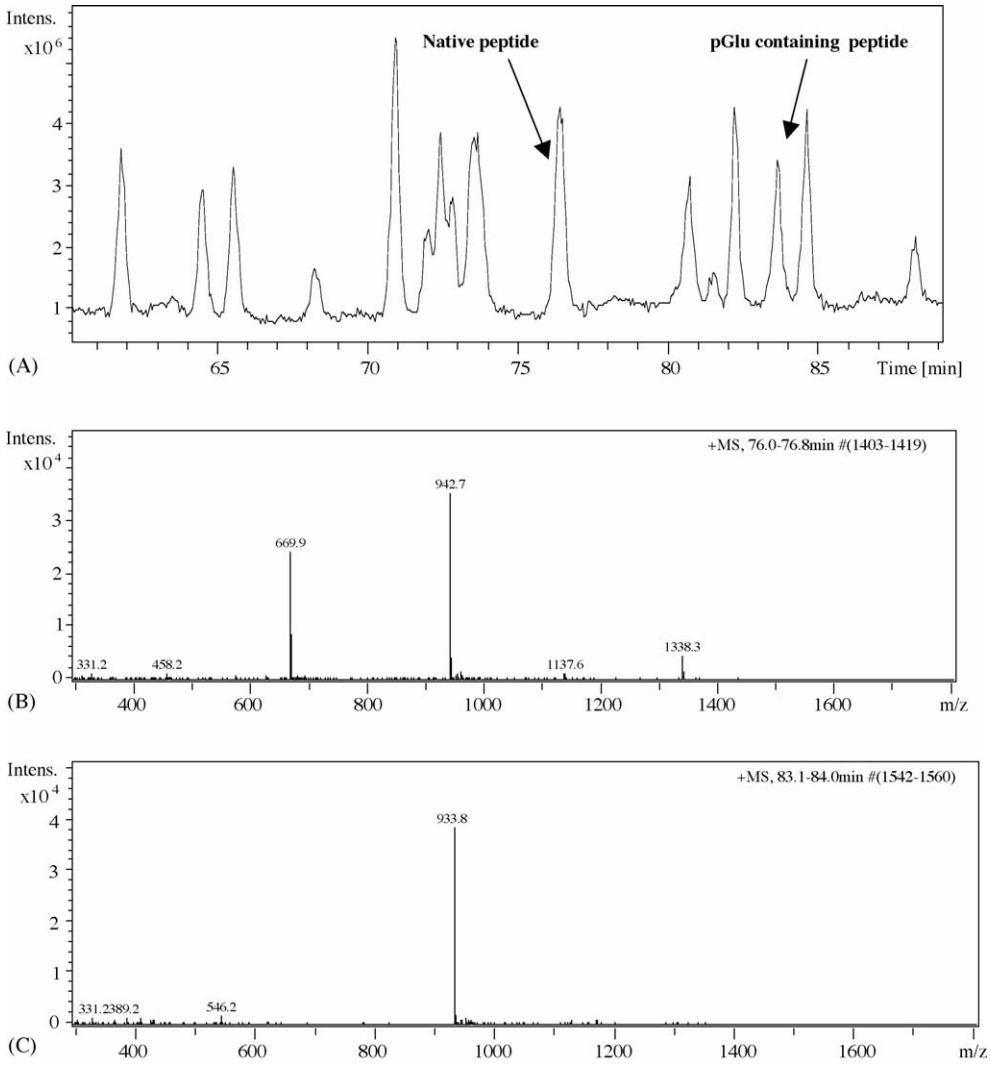

Fig. 1. Peak identification of *N*-pyroglutamyl formation from tryptic peptide mapping LC/MS trap. *N*-Pyroglutamte contained peak was found from stressed formulation sample.

Table 1
N-Pyroglutamate % determined by MSD trap and TOF MS

	Results (pGlu %)				
	pH 4.5	pH 4.7	pH 5.0	pH 5.2	pH 5.5
MSD trap[a]	50	42	38	30	20
QTOF MS[b]	49	43	39	33	27

	Results (pGlu %)					
	Acetate[c]	Propionate[c]	Glycolate[c]	Glutamate[c]	Histidine[c]	Succinate[c]
MSD trap[a]	35	33	40	40	46	45
QTOF MS[b]	33	33	37	37	40	40

Samples were stressed at 45 °C for 3 months.

[a] Data was obtained from peptide mapping by LC/MSD trap. The percentage of pGlu was determined from the integration of the extracted ion chromatogram (XIC) of the native peptide peak mass and pGlu peptide peak mass in the peptide mapping method.

[b] Data was obtained from reduced intact by QTOF MS and based upon the integration of two deconvoluted mass peak area of the native light chain from the purified bulk and modified LC from the stressed samples.

[c] The concentration is 10 mM for all buffers and pH is 5.2.

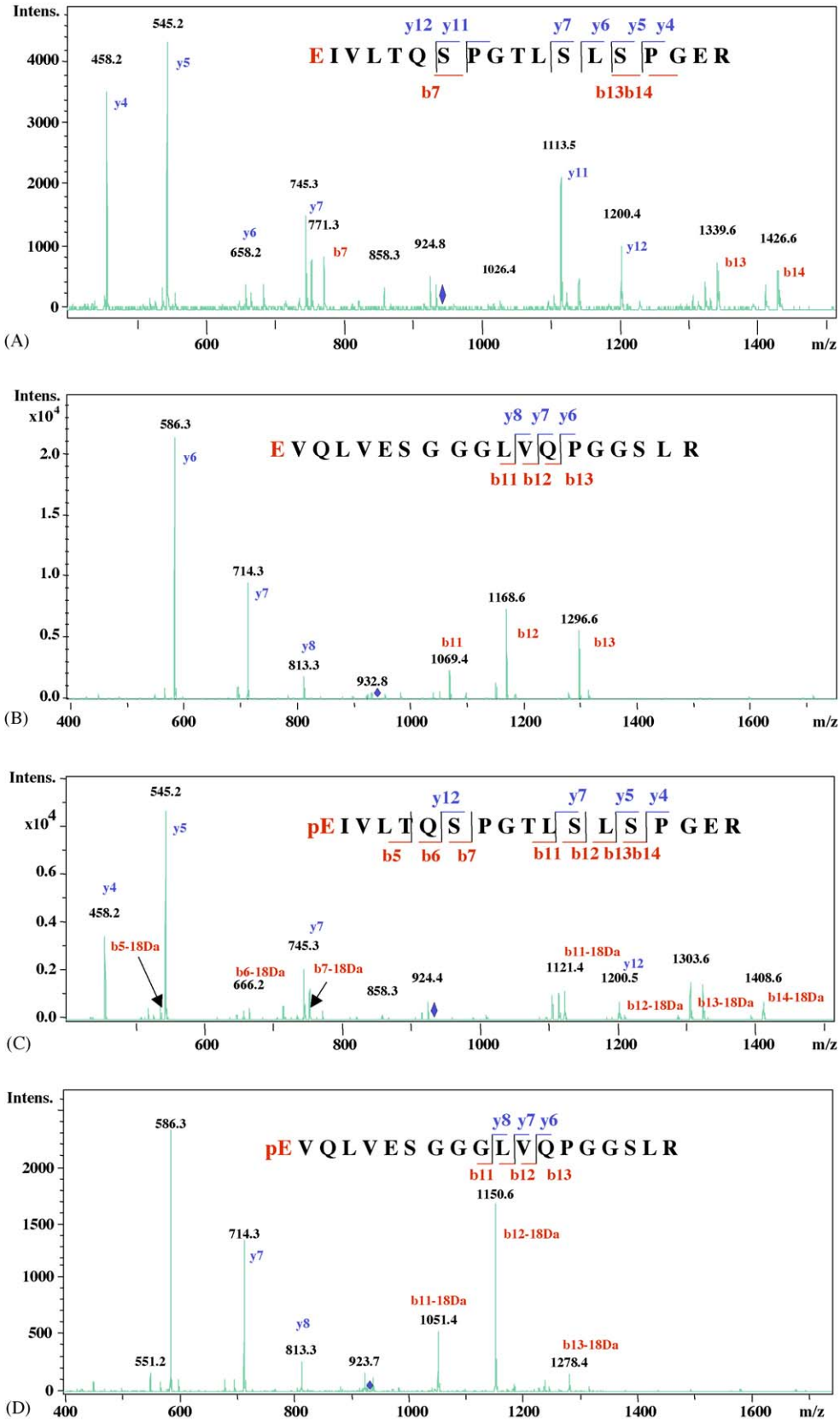

Fig. 2. Light chain and heavy chain MS/MS data of the native *N*-glutamate and *N*-pyroglutamate contained peptides. The native *N*-glutamate contained peptides of LC (A) and HC (B). The *N*-pyroglutamate contained peptides of LC (C) and HC (D).

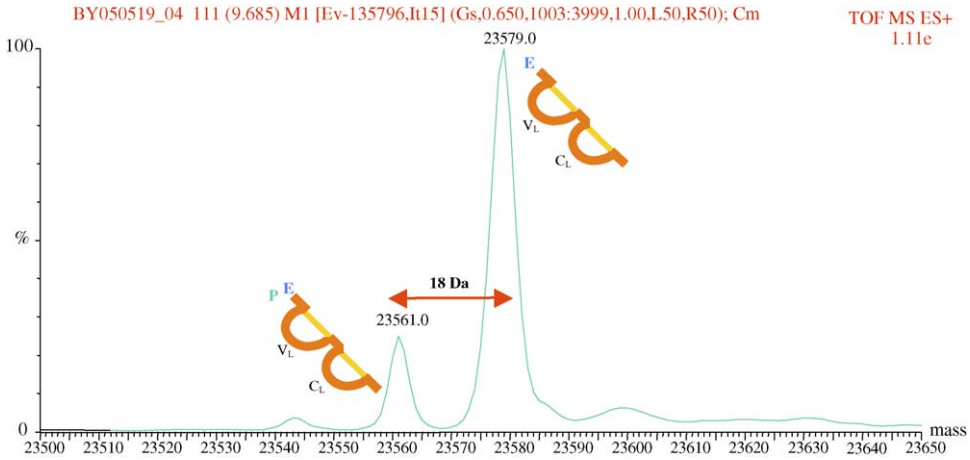

Fig. 3. Deconvoluted mass spectrum of reduced intact sample at 45 °C and 3 months by QTOF MS. Two peaks were determined to be light chain and *N*-pyro light chain that lost 18 Da due to cyclization.

increased with pH. This effect was demonstrated by the LC and HC data.

These data can be used to predict mAb N-terminal Glu storage half-life if the pGlu formation is assumed as a pseudo-first order rate reaction, the rate $[R]_c$ of the pGlu formation is proportional to the concentration of the native antibody.

$$[R]_c = d[pE]/dt = -d[E]/dt = k[E],$$

$$d[E]/([E] + [pE])/dt = -k[E]/[E] + [pE],$$

$$d[E]_r/dt = -k[E]r, \qquad [E]_r = A\exp(-kt),$$

$$\ln[E]_r = -kt + C \tag{1}$$

where $[E]_r$ is relative percentage of N-terminal Glu, [E] and [pE] are concentrations of Glu and pGlu derivatives, respectively. Eq. (1) was used to fit the data to obtain parameters of k (rate constant) and C (ln[percent of native mAb]) at time zero.

The plot of the logarithm of relative percent of N-terminal Glu as a function of time is linear and shown in Fig. 6. The linear regression fit predicts that slope (k) and the intercept (C) equal 0.0003 and equal 0.0101, respectively, for pH 4.5 and 45 °C. Based upon the above equation, the N-terminal Glu half-lives of LC and HC were calculated for other temperature and pH and tabulated in Table 2. Data was not shown for some temperatures since the pGlu formation was insignificant at 37 °C for HC and was undetectable for the LC and HC below 4 °C over 3 months. Data was also collected for 29 °C after 1-year observation with a half-life of 2 years at pH 5.

Buffer, excipient and surfactant were also studied to investigate the possible impact on pGlu formation. The 3-month data

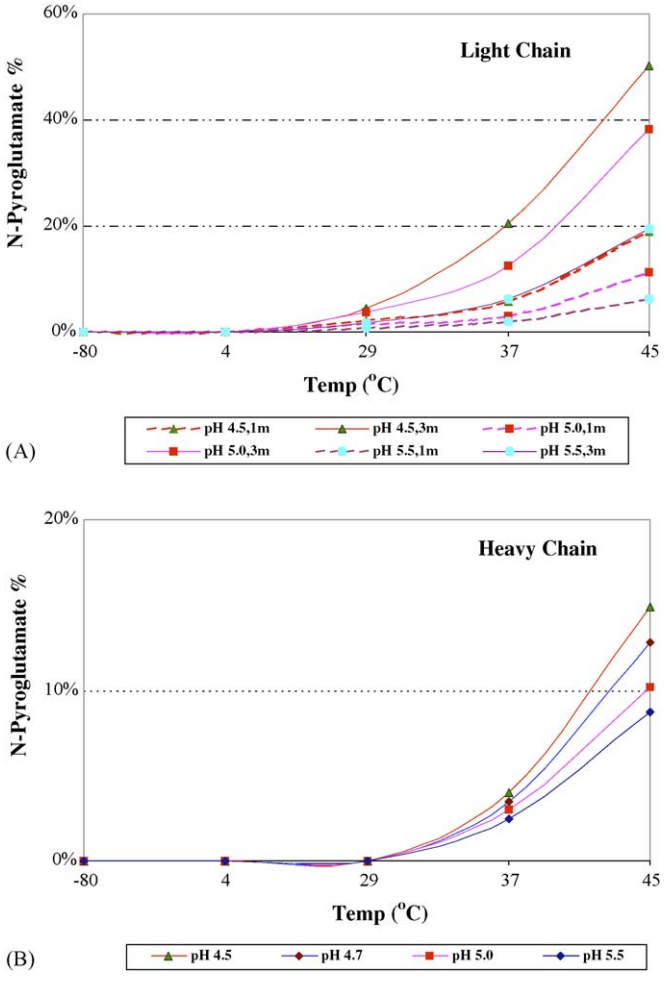

Fig. 4. *N*-Pyroglutamyl formation % of light chain (A) and heavy chain (B) from pH study at 1 and 3 months. Samples stressed at six temperatures, −80, 4, 29, 37 and 45 °C. Data was obtained from peptide mapping by LC/MSD trap.

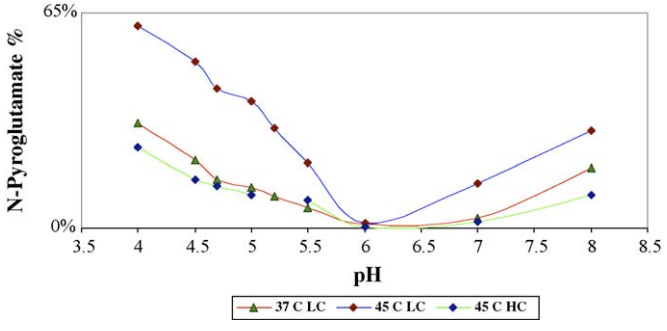

Fig. 5. pH effects on the pGlu formation of the LC and HC of the mAb.

Table 2
N-terminal Glu half-lives[a] of the mAb LC and HC vs. pH

	pH 4.0		pH 5.0		pH 6.0		pH 7.0		pH 8.0	
	LC	HC	LC	HC	LC	HC	LC	HC	LC	HC
37 °C	4.8	NA[b]	16	NA[b]	24	NA[b]	19	NA[b]	11	NA[b]
45 °C	2.4	9.6	4.9	19	9.6	481	9.6	96	4.8	19

The data was collected from the samples stressed at 37 and 45 °C over 3 months.
[a] Half-life ($t_{1/2}$, months) is calculated from linear regression data, the slope (k) and the intercept (C). Data was also obtained at 29 °C after 1-year observation with a half-life of 2 years for pH 5.
[b] Data was not available for the heavy chain at 37 °C since the pGlu formation was undetectable and insignificant.

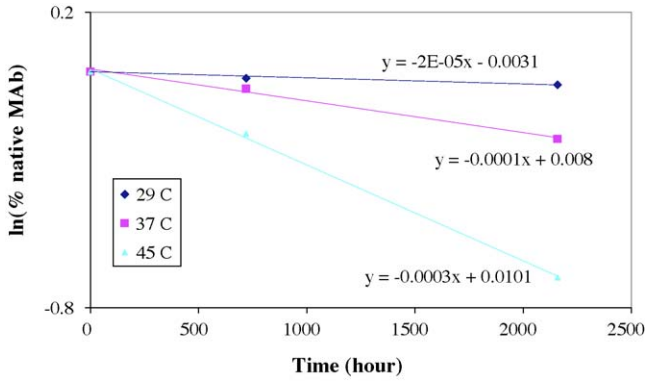

Fig. 6. Logarithm of the native mAb as a function of time at pH 4.5 and over 3 months.

did not show obvious effect as pH, temperature and storage time. Fig. 7 gave the buffer study result after 3-month storage. However, 1-year stability data from 29 °C and pH 5 showed that succinate buffer generated a relatively higher pGlu formation than acetate and histidine buffer. So far, it is unclear if the buffer pK_a and structure may impact the cyclization reaction. It has been reported that weak acids could catalyze the cyclization of Glu [19]. However, the buffer study indicates that the pGlu formation is slightly higher in histidine buffer than in sodium acetate at pH 5.0, suggesting that weak acids do not seem to accelerate the formation of pGlu. Further study may be needed to investigate this hypothesis. Both excipient and surfactant studies did not show any direct impact on pGlu formation with the exception for lactose (Fig. 8A and B). Lactose is a reducing sugar that can be reacted with lysine to form glycated lysine and this glycation was observed in our excipient screen study. This reaction can provide some information on mAb folding/unfolding status, indicating accessibility of a mAb surface. And more, lysine is the primary target for trypsin digestion and glycated lysine is almost non-reactive due to the hindering effect by the attached glycan on its side chain. However, glucose is also reducing sugar and its glycation was also found although glucose did not show significant impact on the cyclization of the mAb. This phenomenon is very interesting and also complicated. Further investigation may be needed to explore the impact of lactose on the cyclization.

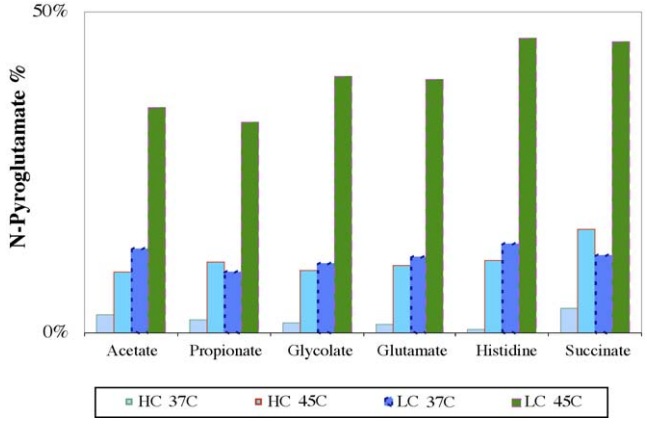

Fig. 7. *N*-Pyroglutamyl formation % of light chain and heavy chain from buffer study at 3 months. Data was calculated from stressed samples at 37 and 45 °C at 3 months.

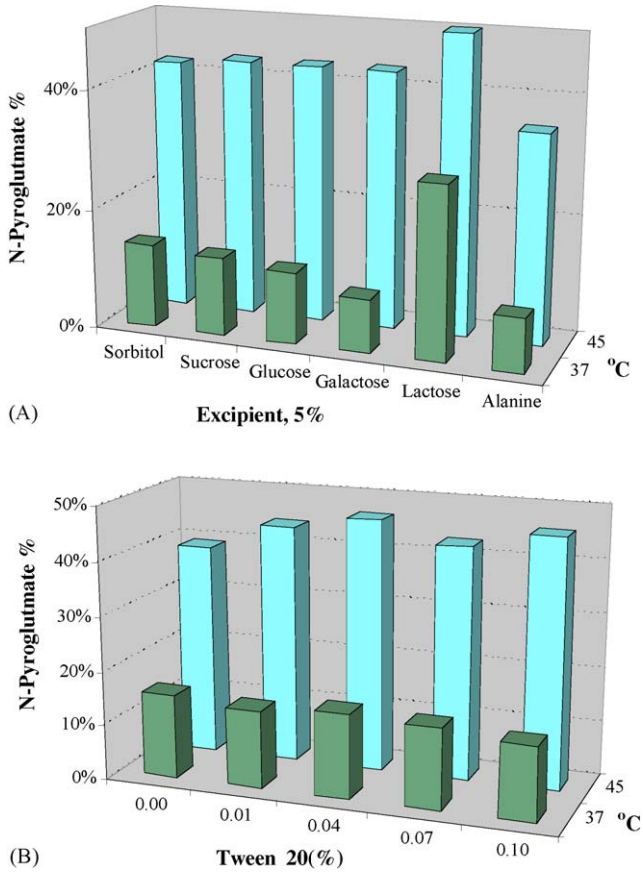

Fig. 8. *N*-Pyroglutamyl formation % of from excipient (A) and surfactant (B) studies at 3 months by LC/MSD trap. Data was calculated from stressed samples at 37 and 45 °C at 3 months.

3.3. Evaluation of IgG2 secondary structure and its biological activities

The literature has proposed that that pGlu from Glu can be an enzymatic reaction because the conversion of Glu to pGlu requires 50 h of incubation at 100 °C and at pH 4 and 10 [12,13]. The cyclization of Glu could hardly occur in relatively mild condition such as within cell culture and during purification and the formulation development process. The mAb in this study showed no detectable pGlu in the bulk materials after purification process. All conversion of Glu to pGlu occurred on shelf storage and grew with time, suggesting that pGlu can be formed under mild condition not through enzymatic reaction in a non-physiological condition. Moreover, this works proves that pGlu was derived from Glu without prior conversion to glutamine.

Both the N-terminus of the LC and HC are located in the framework region 1 and closed to CDR regions, and may potentially impact its bioactivity or potency after conversion to pGlu. If the binding epitope involves the N-terminus, such structural alteration may likely change the binding affinity directly. mAbs are densely packed molecules, cyclization, a modification involves the mAb peptide backbone on both LC and HC, can be reasonably anticipated certain impact on the local binding epitope environment indirectly. Such indirect impact can effect either through long range "linear" backbone adjustment or through the "3D" local bonding network at various levels, from H-bonding to covalent bonding. To isolate the impact of N-terminal cyclization from other physical and chemical degradations, some samples were treated to achieve about 50% N-terminal pGlu conversion with minimal physical and chemical degradations (i.e., aggregation, deamidation, oxidation). Since we did not see any significant tertiary structure change from our near-UV circular dichroism spectroscopy (data not shown), and the mAb contains predominantly β sheet, FT-IR is employed to determine whether or not the conformations of proteins with a broad range of secondary structural compositions are altered. FT-IR spectroscopy is one of the widely used vibrational spectroscopic methods in protein structural analysis, especially sensitive to the small secondary structure change. It is extremely sensitive to the conformational changes of proteins induced by various factors such as temperature, pH, and added chemicals and solvents. Fig. 9 compares the secondary structure of stressed mAb (red solid line) to the non-stressed mAb. A slight change was observed on some β turns around 1676 cm^{-1}. Since the tested sample was stored at 45 °C for 3 months, the β turn change might be caused by thermal stress. So far, we could not observe the significant structural change induced by cyclization. The impact evaluation of the N-terminal cyclization on the biological activity of the mAb remains challenging because it is hardly to obtain purely cyclized sample separately. Some conditions that favor N-terminal cyclization may also favor other physical and chemical degradations as well. This poses an intriguing challenge to isolate the impact of specific degradations on the activity of the affected therapeutic mAbs. Currently, other modifications such as oxidation have been observed in the stressed mAb. Considering that the objective is to minimize degradation of any kind, it immediately becomes clear that awareness of the properties that chemical modifications can have on activity and the ability to quantify them is of practical importance.

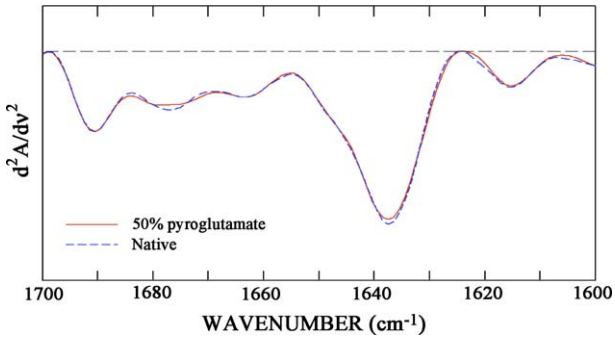

Fig. 9. Data from FT-IR secondary structure study. Comparison of the sample containing approximately 50% *N*-pyroglutamate (red solid line) to the native mAb (blue dotted line). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

4. Conclusions

High conversion of N-terminal Glu to pGlu was observed on the studied mAb in assorted stability studies. This cyclization of Glu was observed and confirmed on the N-terminus of the LC and HC of the protein studied by the two mass spectrometry analytical techniques. Several formulation factors (temperature, time and pH) have been evaluated and the results showed that they significantly affected the formation of *N*-pyroglutamate. Based on the kinetic information presented in this study, the half-lives were calculated at pH 4.5–5.5 for LC and HC. Our data demonstrated that the pGlu of the mAb was formed non-enzymatically during stability studies. This non-enzymatic cyclization of Glu to pGlu of mAbs could be one of the major degradation pathways incurred in the mAb production and storage depending on pH and temperature conditions during the process development. The cyclization of Glu may not introduce significant conformational adaptation of the molecule based upon the preliminary structural change data. Whether or not it may induce further modifications and alter its bioactivity or therapeutic potency is unclear. Therefore, close monitoring of N-terminal pGlu formation can be critical to ensure quality of these mAb therapeutics with N-terminal Glu.

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