In-depth Characterization of Stressed Bispecific Antibody and Assessment of Batch Consistency by UHPLC-HRAM MS Based Peptide Mapping

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Abstract

Purpose: To characterize thermal stress induced potential critical quality attributes (pCQAs) changes and assess batch differences of bispecific antibody (BsAb) samples.

Methods: A Thermo Scientific™ Vanquish™ Flex UHPLC coupled to a Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometry equipped with BioPharma option was used for peptide mapping.

Results: Identification and monitoring of low level thermal sensitive pCQAs of bispecific antibody across different baches via UHPLC-HRAM-MS based peptide mapping.

Introduction

Bispecific antibody (BsAb) has emerged as an important class of biopharmaceutical drugs. Compared to conventional monoclonal antibodies, BsAb characterization is more challenging due to the structure asymmetry and complexity. Therefore, LC-MS based peptide mapping method has become the core technique for BsAb characterization. As publications described, thermal sensitive post-translation modifications (PTMs) such as deamidation, succinimidation and isomerization need to be characterized during the development and monitored thru manufacturing and final lot release since they may affect the stability, safety and efficacy of the biotherapeutics. In this study, several thermal sensitive PTMs were identified as potential critical quality attributes (pCQAs) from a single lot product and subsequently compared across multiple lots to assess batch-to-batch consistency.

Materials and methods

Sample Preparation

Unstressed BsAb: BsAb samples from different lots (sample1-4) were diluted to 1mg/mL using denaturing buffer, then reduced, alkylated and digested with trypsin.

Stressed BsAb: BsAb sample1-4 were thermal stressed by being placed at 50℃ for 24 hours, then followed the same digestion protocol as unstressed samples.

UHPLC Separation

Thermo Scientific™ Vanquish™ Flex UHPLC binary system

Column: Thermo Scientific™ Acclaim™ VANQUISH™ C18 120Å, 2.2 µm, 2.1 x 150 mm, (P/N 071399-V)

UHPLC gradient is listed in Table1.

Flow rate: 0.3mL/min; Solvent A: 0.1%FA, H_2O; Solvent B: 0.1%FA, ACN; Column temperature: 50 ℃

Mass Spectrometry:

Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometry with BioPharma option was used. A ddTOP5 method was employed for data acquisition.

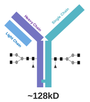
Data Analysis

Data analysis was performed using Thermo Scientific™ BioPharma Finder™ 5.2 software.

Table 1. UHPLC gradient.

Time (min)	Flow (ml/min)	%B
0	0.3	1
5	0.3	1
6	0.3	10
70	0.3	35

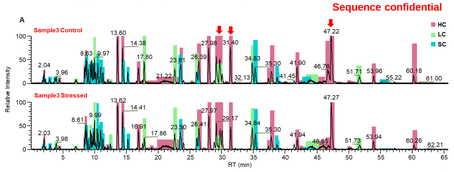
Figure 1. The Schematic of BsAb used in this study.



Results

The BsAb used in this study consists of three chains, one heavy chain (HC), one light chain (LC) and one single chain (SC), as shown in Figure1. The goal for this study is to find out thermal sensitive hotspots of this molecule by characterize thermal stressed sample of one lot first, then apply these pCQAs for batch consistency assessment of samples from other lots. Figure2 displays BPC profile and sequence coverage of sample3. Over 96% sequence coverage is obtained for all samples. Uncovered regions are those with continuous K and R that generated short peptides by trypsin digestion, which are not suitable for MS2 identification but can be identified with MS1. As labelled in Figure2, abundance of some peaks changed before/after thermal stress, indicated thermal sensitive peptides were eluted at these time.

Figure 2. BPC profile and sequence coverage map of sample3. A, BPC profile of both control and stressed sample3. read arrows indicate peaks of thermal sensitive peptides. B, sequence of sample3 control.



Proteins	Number of MS Peaks	MS Peak Area	Sequence Coverage	Abundance (mol)
1:HC	858	47.7%	97.1%	41.98%
2:LC	259	15.2%	98.6%	37.33%
3:SC	346	12.0%	96.8%	20.70%

Thermal sensitive hotspots identification

After comparison of stressed and unstressed sample peptide mapping results, pCQAs such as deamidation, succinimidation and isomerization at specific sites were identified and significant changes of modification% were observed. Take Sample3 as an example, the deamidation% at N289 HC (N315 SC) in stressed sample3 is 0.125% vs 0.056% in unstressed sample3; the succinimidation% at N289 HC (N315 SC) in stressed sample3 is 0.16% vs 0.10 % in unstressed sample3; the isomerization% at D283 HC (D309 SC) in stressed sample3 is 1.65% vs 0.11% in unstressed sample3. Oxidation% also increased in all thermal stressed samples. These changes provide clearly evidence of thermal sensitive hotspots in this BsAb. XIC peak, MS2 spectra and fragment coverage map of modified FNWYVDGVEVHN(289)AK of heavy chain in sample3 can be found in Figure3. High sensitivity and mass accuracy provided by Orbitrap HRAM mass spectrometry enables identification and relative quantitation of trace(<0.1%) PTMs. Table2 lists all thermal sensitive hotspots identified in sample3.

Figure4-7 and Table3-6 show the identification and relative quantitation results of each pCQAs in different samples, all modification% are averaged from three replicate injections. Figure8 and Table7 show the HC(SC) C-terminal lysine truncation% and RSD in all samples, Figure9 and Table8 display HC(SC) N-glycosylation% and RSD in all samples. As expected, lysine truncation% and N-glycosylation% weren't affected by thermal stress.

Table2. Thermal sensitive hotspots identified in sample3. stressed/control ratios are also listed in the table.

Deamidation		Succinimidation		Isomerization		Oxidation	
Site	Ratio	Site	Ratio	Site	Ratio	Site	Ratio
N289 HC(N315 SC)	2.23	N289 HC(N315 SC)	1.56	D283 HC(D309 SC)	14.64	M255 HC(M281 SC)	1.40
N318 HC(N344 SC)	1.42	N318 HC(N344 SC)	1.10			M431 HC(M457 SC)	1.61
N418 HC	1.12	N437 HC(N463 SC)	1.13				
N31 HC	1.28						

Figure 3. MS2 spectra and fragment coverage map of FNWYVDGVEVHN(289)AK of heavy chain in sample3. This peptide also exists in single chain. A, XIC/MS2 spectra/fragment coverage map of deamidated peptide in control/stressed sample3. B, XIC/MS2 spectra/fragment coverage map of succinimidated peptide in control/stressed sample3.

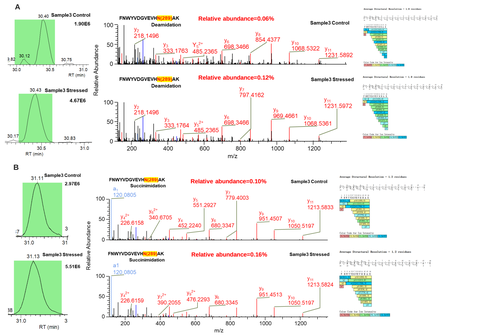


Figure 4. Deamidation% in all samples.

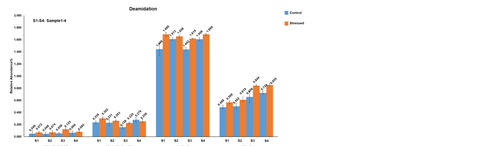


Table3. Deamidation% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
N289 HC(N315 SC)Avg.Rel%	0.05	0.07	0.05	0.07	0.06	0.12	0.07	0.08
RSD	5.11%	1.67%	2.58%	2.88%	1.23%	2.01%	2.98%	1.95%
N318 HC(N344 SC)Avg.Rel%	0.24	0.30	0.23	0.26	0.16	0.22	0.28	0.26
RSD	2.20%	2.30%	5.03%	1.31%	3.15%	1.15%	4.02%	3.38%
N364 HC Avg.Rel%	0.20	0.21	0.21	0.22	0.11	0.11	0.21	0.22
RSD	1.44%	1.13%	2.26%	1.01%	6.94%	2.47%	2.03%	1.21%
N418 HC Avg.Rel%	1.45	1.69	1.61	1.66	1.44	1.62	1.61	1.69
RSD	10.35%	3.32%	2.47%	2.12%	3.03%	0.80%	2.83%	1.58%
N31 HC Avg.Rel%	0.49	0.57	0.50	0.61	0.66	0.84	0.72	0.85
RSD	3.33%	2.74%	4.56%	3.76%	9.70%	1.67%	2.83%	2.24%

Figure 5. Succinimidation% in all samples.

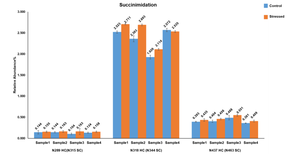


Table 4. Succinimidation% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
N289 HC(N315 SC)Avg.Rel%	0.14	0.15	0.14	0.16	0.10	0.16	0.13	0.16
RSD	4.25%	1.79%	1.55%	2.12%	1.92%	7.36%	1.62%	1.62%
N318 HC(N344 SC)Avg.Rel%	2.52	2.71	2.36	2.69	1.93	2.11	2.57	2.53
RSD	2.54%	1.44%	7.22%	2.14%	4.69%	2.08%	3.88%	2.17%
N437 HC (N463 SC)Avg.Rel%	0.39	0.44	0.40	0.46	0.49	0.55	0.36	0.41
RSD	1.14%	2.88%	2.77%	1.78%	5.26%	2.17%	0.71%	2.14%

Figure 6. Isomerization% in all samples.

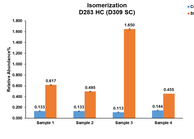


Figure 7. Oxidation% in all samples.

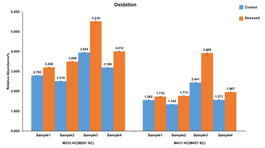


Table 5. Isomerization% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
D283 HC(D309 SC)Avg.Rel%	0.13	0.62	0.13	0.49	0.11	1.65	0.14	0.46
RSD	1.92%	0.63%	1.03%	1.41%	2.45%	0.75%	0.32%	1.68%

Table 6. Oxidation% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
M255 HC(M281 SC)Avg.Rel%	2.79	3.21	2.51	3.50	3.95	5.52	3.26	4.01
RSD	0.51%	2.51%	1.57%	2.58%	2.59%	8.30%	1.80%	1.54%
M431 HC(M457 SC)Avg.Rel%	1.56	1.74	1.34	1.77	2.44	5.52	1.57	1.97
RSD	3.62%	2.96%	2.02%	1.69%	4.84%	8.30%	2.46%	2.11%

Assessment of Batch Consistency

With thermal sensitive pQAs successfully identified and quantified, then we compared these pCQAs in different lots of samples. The modification% changing trend before/after thermal stress is different among different lots. the abundance% of deamidation at N289 HC (N315 SC) in stressed sample 1-4 are 0.071%, 0.073%, 0.120% and 0.083%; the succinimidation% at N318 HC (N344 SC) in stressed sample 1-4 are 2.711%, 2.693%, 2.114% and 2.535%, and the isomerization% at D283 HC (D309 SC) in stressed sample 1-4 are 0.617%, 0.495%, 1.650% and 0.455% respectively. The behavior of sample3 is obviously different from others.

The HC(SC) C-terminal lysine truncation% is thermal stable, however, in sample 1 and 2, the lysine truncation% are around 75% while in sample 3 and 4, the lysine truncation% are around 85%, as shown in Figure8 and Table7. This batch difference may induce charge heterogeneity.

The most abundant N-glycoform is A2G0F, which is around 80% for all stressed/unstressed samples since N-glycosylation is not thermal sensitive. We also observed over 6% of A3G0F in sample3 and 4, but in sample1 and 2, the percentage of A3G0F are between 4.5%-5%. Besides the batch difference, A3G0F% of this sample is higher than most of mAbs. Batch difference of N-glycosylation distributions can be observed in Figure9 and Table8.

Figure 8. HC(SC) C-terminal Lysine Truncation% in all samples.

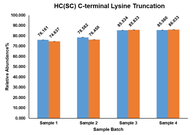


Table 7. HC(SC) C-terminal Lysine Truncation% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
Avg.Rel.%	76.16	74.64	78.58	76.45	85.53	85.83	85.56	86.03
RSD	1.42%	0.48%	5.77%	0.59%	0.17%	0.29%	0.80%	0.52%

Figure 9. HC(SC) N-glycosylation% in all samples.

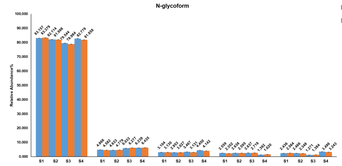


Table 8. HC(SC) N-glycosylation% and RSD in all samples.

	Sample1		Sample2		Sample3		Sample4	
	Control	Stressed	Control	Stressed	Control	Stressed	Control	Stressed
A2G0F Avg. Rel.%	83.16	83.38	82.11	82.00	79.54	78.98	82.78	81.86
RSD	0.25%	0.11%	0.38%	0.36%	0.39%	0.38%	0.23%	0.19%
A3G0F Avg. Rel.%	4.87	4.58	4.63	4.78	6.03	6.28	6.24	6.43
RSD	4.29%	3.59%	1.62%	4.04%	1.54%	1.19%	1.89%	0.20%
A1G0F Avg. Rel.%	3.10	3.13	2.95	3.03	3.47	3.13	4.45	4.14
RSD	2.24%	2.24%	2.54%	2.60%	1.22%	1.15%	3.04%	0.83%
A3G1F Avg. Rel.%	2.56	2.35	2.64	2.60	2.64	2.72	1.39	1.62
RSD	2.39%	2.39%	3.15%	2.47%	4.85%	2.46%	3.03%	4.31%
M5 Avg. Rel.%	2.44	2.56	2.47	2.35	1.27	1.38	3.50	3.24
RSD	2.47%	3.07%	5.13%	1.69%	2.15%	2.12%	2.94%	3.66%

Conclusions

- In this study, we demonstrated the confident identification and reproducible relative quantification of low level thermal sensitive pCQAs, such as deamidation and succinimidation of N289 HC (N315 SC), isomerization of D283 HC (D309 SC), of a bispecific antibody across different baches via HRAM-MS based peptide mapping approach.
- Batch differences were observed for some modifications/variants, such as deamidation%, succinimidation%, isomerization and lysine truncation%.

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