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Characterization and Comparison of Disulfide Linkages and Scrambling Patterns in Therapeutic Monoclonal Antibodies - Using LC-MS with Electron Transfer Dissociation

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Summary

The disulfides in three monoclonal antibodies (mAb), the anti-HER2, anti-CD11a, and GLP-1 with IgG4-Fc fusion protein, were completely mapped by LC-MS with the combination of ETD and CID fragmentation. In addition to mapping the 4 inter- and 12 intra-chain disulfides (total 16), the identification of scrambled disulfides in degraded samples (heat-stress) was achieved. The scrambling was likely attributed to an initial breakage between the light (Cys 214) and heavy (Cys 223) chains in anti-HER2, with the same observation found in a similar therapeutic mAb, anti-CD11a. On the other hand, the fusion antibody, with no light chain but containing only 2 heavy chains, generated much less scrambling under the same heat-stressed conditions. The preferred sites of scrambling were identified, such as the intra-chain disulfide for CxxC in the heavy chain, and the C194 of the heavy chain pairing with the terminal Cys residue (C214) in the light chain. The inter-chain disulfides between the light and heavy chains were weaker than the inter-chain disulfides between the two heavy chains. The relative high abundance ions observed in ETD provided strong evidence for the linked peptide information, which was particularly useful for the identification of the scrambled disulfides. The use of SDS-PAGE helped the separation of these misfolded proteins for the determination of scrambled disulfide linkages. This methodology is useful for comparison of disulfide stability generated from different structural designs, and providing a new way to determine the scrambling patterns, which could be applied for those seeking to determine unknown disulfide linkages.

Introduction

Therapeutic monoclonal antibodies (mAb) can bind to specific epitopes on surface receptors and disrupt the interaction between signaling molecules and surface receptors, thereby inhibiting cancers or other inflammatory diseases.[1,2] For example, anti-HER2 mAb (Herceptin) binds to the HER2 receptor, disrupting the receptor complex on the breast cancer cell surface and thus blocking the corresponding signal transduction cascade. The binding domain (Fab) and IgG1 construct (Fc) of Anti-HER2 mAb is connected with disulfides, which link light and heavy chains, and also the two heavy chains (see Figure 1A). There are a total of sixteen disulfides in one anti-HER2 mAb molecule, twelve intra- and four inter-chain disulfides. Among the four inter-chain disulfides, two link together the heavy chains and the other two connect the light and heavy chains. The C-terminal end of the light chain (Cys 214) forms a disulfide bond with Cys 223 in the heavy chain, which thus connects the light and heavy chains. Cys 229 and Cys 232 in one heavy chain link with the other heavy chain at the same Cys 229 and Cys 232 sites to form two parallel inter-chain disulfides between the two heavy chains. These four polypeptide chains (two heavy and two

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light chains) are connected by these four inter-chain disulfide bonds to form a tetramer, which is required for mAb to function effectively,[3–8] and incomplete formation of disulfide bonds could cause loss of drug efficacy.[9,10] Thus, it is important to characterize all these disulfides and establish the presence of correct connectivity to assure drug function and quality.

The disulfides in mAb, similar to other classes of protein, are conventionally characterized by Edman sequencing and tryptic mapping with reduced and non-reduced conditions.[4,7,11,12] Due to its superior selectivity and sensitivity, mass spectrometry (MS) coupled with liquid chromatography has recently become the choice for characterization of disulfides.[13–16] The MS approach often involves the characterization of disulfide-dissociated peptides with reduction and then the determination of disulfide bonds without reduction through collision induced dissociation (CID).[7,11,12,17] However, these approaches can be tedious and may not be feasible for multiple intertwined disulfides in one peptide. Recently, Wu et al used online LC-MS with electron-transfer dissociation (ETD) approach to successfully identify disulfide linkages and multiple intertwined disulfides in several recombinant therapeutic proteins.[18,19]

It has been reported that disulfide scrambling can occur, particularly at alkaline pH or in the presence of free cysteine residues.[7,20–22] However, the determination of the scrambling sites in mAb has not been studied extensively but is of importance in determining product stability as well as in selection of proper formulation. In this report, the disulfides of three different mAb, anti-HER2 and anti-CD11a (both with IgG1 construct) (Figure 1A), and a fusion protein (GLP-1 with IgG4 Fc domain) (Figure 1B), are characterized with the LC-MS with ETD in combination with efficient separation and multi-enzyme approach. This detailed characterization pinpoints the scrambled sites, which could then shed light on the relative strength of specific disulfides, the preferred sites of scrambling, and the scrambling process during a heat-stress study.

Experimental

Materials

Three therapeutic monoclonal antibodies were used in this study: (1) anti-HER2 (Herceptin, Genentech, South San Francisco, CA), a liquid formulation product (22 μg/μL); (2) anti-CD11a (Raptiva, Xoma, Berkeley, CA), a lyophilized powder; (3) GLP-1 with IgG4 Fc fusion protein (CNTO 736, Centocor R&D, Radnor, Pennsylvania), a liquid formulation product (10 μg/μL). Herceptin and Raptiva were the gift from Genentech, and CNTO 736 was the gift from Centocor. Trypsin (sequencing grade) was purchased from Promega (Madison, WI). Guanidine hydrochloride, ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IAA), and formic acid (FA) were from Sigma-Aldrich (St. Louis, MO). LC-MS grade water was from J.T. Baker (Phillipsburg, NJ), and HPLC grade acetonitrile from ThermoFisher Scientific (Fairlawn, NJ). Pre-cast NuPAGE 4–12% Bis-Tris SDS-PAGE gels, NuPAGE MES SDS running buffer (20X), NuPAGE LDS sample buffer (4X), and SimplyBlue SafeStain were all from Invitrogen (Carlsbad, CA).

Heat-stressed conditions

For the heat-stressed stability study, an aliquot of 20 μg monoclonal antibody (i.e. 2 μL) was mixed with 5 μL NuPAGE LDS Sample Buffer (4X) (pH 7.8), and 13 μL NuPAGE MES SDS Running Buffer (1X) to achieve the final volume of 20 μL. The mixtures were incubated at 70°C for 0, 10, 15, 20, 25, 30, and 35 min, separately. For comparison purposes (under reduced conditions), an aliquot of 20 μg monoclonal antibody (i.e. 2 μL) was mixed with 2 μL DTT, 5 μL NuPAGE LDS sample buffer (4X), and 11 μL NuPAGE MES SDS running buffer (1X) to achieve the final volume of 20 μL. The mixtures were incubated at 90°C for 10, 15, 20, 25, 30, and 35 min, separately. After incubation, the mixtures (20 μL)

were loaded onto an SDS-PAGE gel (4–12% Bis-Tris polyacrylamide) at 200 V for 40 min and then stained with Coomassie blue.

Enzymatic digestion

In-gel digestion: bands of interest were excised and destained by alternating use of acetonitrile and 0.1 M ammonium bicarbonate (pH 7.8) until no visible color was present. The destained gel pieces were completely dehydrated with acetonitrile and then dried in a SpeedVac (Labconco, Centrivap Cold Trap). Trypsin or Lys-C (1:50 w/w) was added to just cover the dried gel pieces. After 45–50 min incubation at 4 °C, the remaining supernatant was removed and buffer without trypsin was added to cover gel pieces for overnight (~12 hrs) at 37°C. The digested peptides were then extracted with 5% formic acid and ACN (1:2, v/v) and then dried down until only 10 μL were left (if not, the samples were reconstituted to 10 μL for subsequent analysis).

LC-MS

An aliquot of 5 μL of the digest was injected onto a self-packed C18 column (Magic C18, 200Å pore and 5 μm particle size, 75 μm i.d. ×15 cm) (Michrom Bioresources, Auburn, CA). Mobile phase A was 0.1% formic acid in water and mobile B was 0.1% formic acid in acetonitrile. The peptides were eluted at 200 nL/min using 2% B to 65% B in 65 min, then from 65% B to 80 % B for 10 min. The MS experiments were performed on an LTQXL with ETD mass spectrometer (Thermo Fisher Scientific, San Jose, CA). The instrument was operated in a data-dependent mode: the first survey MS (scan 1) from m/z 400 to 1900 followed by three consecutive ion activation steps: CID-MS^2 (scan 2), ETD-MS^2 (scan 3), and CID-MS^3 (scan 4). The CID-MS^2 and ETD-MS^2 scan steps were performed on the same precursor ion (see the details in our previous paper (17)). The precursor ions of these disulfides were also measured by an LTQ-FT MS instrument (ThermoFisher Scientific).

Results and Discussion

In this section, the digestion strategy, LC-MS method, and principles for assignment of scrambled disulfides will be first described, followed by the analysis of disulfides for three different mAb in a heat-stressed study.

Digestion Strategy

Identification of a single disulfide linkage is straight forward because there is usually only one possibility for connection. So, enzymes, which can cut proteins to the peptides containing only single disulfide, are desired. In addition, peptide sizes are preferred at 1 to 5 kDa since the recovery and electrospray ionization efficiency can be a problem for larger peptides, while smaller peptides may not retain well on a typical reversed phase column.[4] Thus, the selection of proper enzymes or the use of multiple enzymes should be considered for the size adjustment. In addition, the presence of a disulfide bond may prevent cleavage of neighboring residues (steric hindrance). Therefore, during data analysis, additional miscleavages need to be considered. In this work, trypsin was found to be sufficient for in-gel digestion except for the need to use Lys-C for the analysis of a small disulfide-linked peptide (see Table 1). However, Lys-C plus trypsin digestion is preferred than trypsin only (produced less miscleavages) for in-solution digestion (e.g. protein digestion without SDS-PAGE). The reason could be that the cleavage of the disulfide-linked proteins may need Lys-C to trim the size for reducing steric hindrance first.

LC-MS with CID and ETD

The disulfide-linked peptides are characterized by the combination of CID and ETD; specifically, the disulfides are preferentially dissociated into two polypeptides by ETD while CID generates mainly peptide backbone cleavage (with the disulfides intact). This strategy can be further enhanced by CID-MS^3 (after ETD) on the dissociated peptides or the charge-reduced species if necessary, as described in our previous papers.[18,19]

Disulfide Assignment

When two polypeptides are linked by a disulfide bond, the assignment of such peptides could not be achieved by typical approaches such as using either Sequest or Mascot software. Often, the precursor ions with the anticipated linked-peptides could be used for initial screening. The likely candidates (from the matched masses) can then be further confirmed by their fragmentation ions produced under either CID or ETD or even CID-MS^3 fragmentation.

As an example, the identification of a heavy chain disulfide, linkage between T35 and T40 of anti-HER2, is shown in Figure 2. The disulfide was still linked together after fragmentation in CID (Figure 2A), while the disulfide was broken into two separate polypeptides by ETD (P1 and P2 in Figure 2B). The dissociated peptide, such as P2 (or T40) could then be further identified by CID-MS3 (Figure 2C). The high intensity of P1 and P2 in ETD (preferred cleavage) was likely due to the free electron was more easily captured by sulfurs (i.e. disulfides) than by peptide backbones (i.e. N-Ca bonds) during the electron capture or transfer process (see our previous references for more details).[18,19]

Heat-stressed study

Anti-HER2 samples were heated (without reduction with DTT) at 70°C for 0, 10, 15, 20, 25, 30, and 35 min, and then separated by SDS-PAGE (Figure 3 left panel). As shown, the intensities of additional bands were increased with longer heating times. However, these same samples under reduction (DTT treatment) were collapsed into two major bands (heavy and light chains) regardless the heating time. Thus, the additional bands, while disappearing after reduction, could be related to mAb structures with different combinations of disulfide linkages.

Scrambled disulfides

The disulfides with the known and expected linkages were identified in band A1 at the 0 time point (see Table 2). However, the additional bands generated at different heating times (i.e. A1a, A1b, A1c, and etc in Figure 3A) were found to contain different disulfide connectivity. As an example, a scrambled disulfide was identified in band A1d. In this band, in addition to the anticipated disulfide linkage, T35 with T40, the scrambled linkage of T35 with T11 was identified. The disulfide-linked peptide was dissociated into two polypeptides by ETD (T11 or P1 and T35 or P2 in Figure 4B). The observation of two relative high abundance polypeptides (T11 and T35) provided strong evidence of these two linked peptides, which was further confirmed by CID-MS3 (Figure 4C) or CID-MS2 (Figure 4A). However, for the robust identification of scrambled disulfides, a combination of all possible cysteine-containing tryptic peptides for the mAb sequence need to be considered.[23] After making a mass list of all possible combinations with different charge states, the likely precursors were then further examined by their corresponding ETD and CID fragmentation ions. The high abundant ETD ions were typically examined first since the disulfide-dissociated peptides were often produced with relative high intensities. If these disulfide-dissociated peptides in ETD matched with any of the cysteine-containing tryptic peptides, their corresponding CID-MS2 and CID-MS3 (after ETD) were then examined for further

confirmation. Overall, the ETD spectrum provided an initial match for the presence of scrambled disulfides from the list of potential precursor ions. The examples of the light and heavy chain scrambling were displayed in the Supplemental Material Two examples of the light chain scrambling in band A2 were displayed in the Supplemental Material, see Figure S1 (A and B) for the light chain scrambling in band A2 and Figure S2 (A and B) for the heavy chain scrambling in band A1a. For simplicity, only the ETD spectra were displayed for the identification. The identification of different combinations of light and heavy chains (along with their corresponding disulfide linkages and major scrambled disulfides) in these additional bands was listed in Table 3. These assignments were based on the disulfides and peptide sequences found at their corresponding gel bands.

From these heat-stressed studies, the initiation of the scrambling could be proposed to occur at the linkage between the light and heavy chain. Under storage at mild temperatures (i.e. 4 °C for 3 yrs in a liquid formulation), a minor band at the light chain position was observed (band A2 at 0 time point in Figure 3A). This minor band was identified by peptide sequence as a breakage of light chain from the intact mAb. There is only one linkage between the light (Cys 214) and heavy chains (Cys 223) (see Figure 1A). Thus, this breakage should produce the nuclei (free cysteine) for initiation of scrambling, which was manifested with different combinations of scrambled disulfides and increased more with longer heating times.

Stability for other types of mAb

As examples of other therapeutic mAb, anti-CD11a in a lyophilized powder form was investigated with the same analytical approach. Except for the presence of different amino acid sequences in the Fab region, anti-CD11a has the same numbers and types of disulfides as anti-HER2 (same IgG1 type). After heating, anti-CD11a generated a very similar band pattern as anti-HER2 under non-reduced conditions (see Figure 5A), and similar scrambling was also found in the additional bands (see Tables S1A and S1B in the Supplemental Material). Another fusion antibody was also investigated under the same conditions. In this fusion antibody, the light chain was removed and the heavy chain in the Fab region was replaced with a GLP-1 sequence (CNTO 736).[24] The heavy chains in the IgG4 Fc domain have the same types and numbers of two parallel inter-chain disulfides to connect the two heavy chains and four intra-chain disulfides within the heavy chains (see Figure 1B). After a similar heating stability study, the additional bands observed in SDS-PAGE were significantly reduced for the fusion antibody (compare Figure 5B to Figure 5A). This reduction attributed again to the absence of light chains, which likely initiated the scrambling in anti-HER2 and anti-CD11a. The results also suggest that the inter-chain disulfides between the light and heavy chains are weaker than the inter-chain disulfides between the two heavy chains. In this heat-stress study, the dissociated heavy chains migrated at the molecular weight corresponding to one half size of the intact molecule (band 2 vs. band 1 in Figure 5B). This dissociated heavy chain was found to contain an intra-chain disulfide bond connected between the Cys 229 and Cys 232 (see Figure 6). The assignment was based on the accurate precursor mass measurement (the loss of 2H from the peptide containing 2 cysteines), as well as the corresponding fragmentation patterns of the precursor ion in CID-MS2 (Figure 6A) and ETD-MS2 (Figure 6B). As shown, the fragment ions in this peptide were derived from outside of the disulfide ring. Particularly, the fragmentation at the proline residues by CID (y ions) was observed with high abundance near but not within the intra-chain disulfide. The disulfide exchange from inter- to intra-chain disulfide in the CxxC region of the heavy chain could also be found in anti-HER2 and anti-CD11a mAb, the latter two being IgG1 type Fc as compared to the IgG4 type for the fusion protein (see Figures S3 and S4 in the Supplemental Material). For the fusion protein, this disulfide exchange in the CxxC seemed to be the preferred scrambled site as compared to other scrambled sites (judged by the 1 to 2 order magnitude higher intensity; see Supplemental

Material in Tables S2A and S2B). Since different peptides could have different ionization efficiency, we also used relative comparison to compare samples at different heating times (0 to 35 minutes as shown in Figure 5B). Indeed, the relative ratio of the intra-chain disulfide of CxxC (ratio against an internal peptide without disulfides) was increased much faster and higher than any other scrambling disulfides with longer heating time. For mAb with the light and heavy chains (anti-HER2 and anti-CD11a), the scrambling at CxxC was then the second most preferred site relative to the disulfide linkage between the light and heavy chains. The formation of the intra-chain disulfide (CxxC) caused the two heavy chains breaking into a single heavy chain (molecular weight shift as shown in the band 2 of Figure 5B or band A1d of Figure 3A). For this scrambled site, neither the two free cysteines nor the crosslink (i.e. between C229 of the heavy chain 1 with C232 of the heavy chain 2 and C232 of the heavy chain 1 with C229 of the heavy chain 2) could be observed.

From characterization and comparison of these scrambled disulfides, a focus should be placed on the disulfide connection between the light and heavy chains, and also the disulfide connection between the two heavy chains in the manufacturing processes or during formulation development. Any inconsistent recovery (intensity) or retention time for these two disulfide-linked peptides would provide a warning signal for scrambling. A time course study with relative comparison, as shown here, should provide a good indication of disulfide stability for mAb development. In addition, any artifact (e.g. scrambling caused by digestion conditions) can also be determined and eliminated from the time course study.

Conclusions

Our method successfully identified the scrambled disulfides with linkage sites in mAb. The scrambling that we observed in our heat-stressed study was likely due to the initial breakage between the light (Cys 214) and heavy (Cys 223) chains. For proteins containing only Fc region, the breakage of inter-chain disulfides between the two heavy chains (CxxC) was facilitated by the formation of the intra-chain disulfide linkage.

In this method, the combination of ETD with CID fragmentation successfully mapped the correct and scrambled disulfides of three different mAb, with the relative high abundant ions observed in ETD providing strong evidence of the linked peptide. The use of SDS-PAGE to separate the misfolded proteins helped to decide the scrambled disulfide linkages. The relative strength of disulfides with their correct linkages and preferred scrambling sites could be examined from a time course study.

The higher order structures such as disulfide mapping in proteins are usually difficult task to characterize comprehensively because of the technique challenge. The new ETD mass spectrometry technology, developed recently,[25] allowed us to investigate this important phenomenon. The method in this study provided a new way to determine the scrambling patterns, which could be applied for those seeking to determine unknown disulfide linkages. This method also set the stage to compare the higher order structure such as disulfide linkages for biotechnology industry at different development stages or biosimilar comparison.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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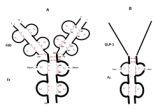

Figure 1.
The disulfide structures of anti-HER2 mAb (A), and GLP-1 fusion mAb (B).

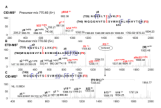

Figure 2.
Using CID-MS^2 (A), ETD-MS^2 (B), and CID-MS^3 (C) to analyze a disulfide-linked peptide (anti-HER2).

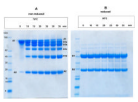

Figure 3.
Heat-stressed study for mAb (anti-HER2) using SDS-PAGE separation with non-reduced (A) and reduced (B) conditions.

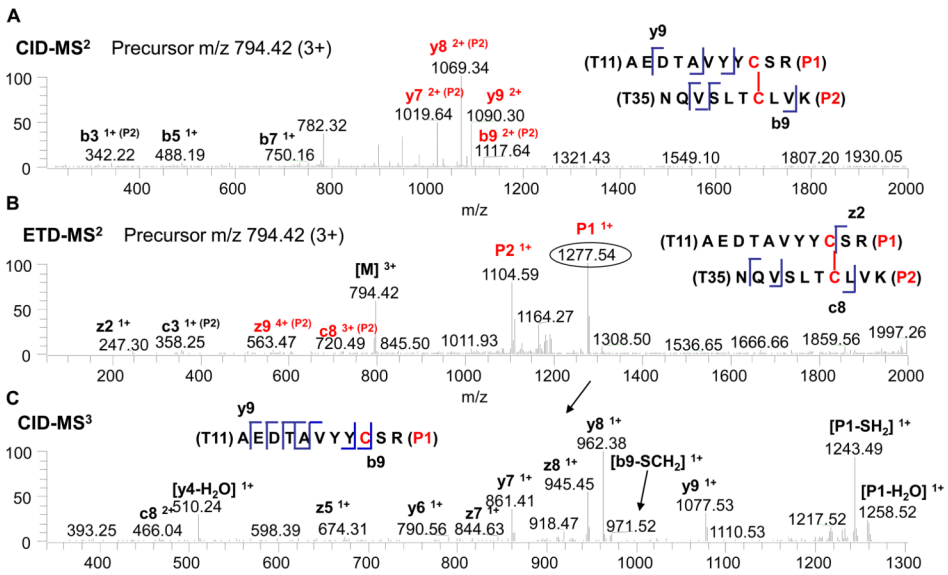

Figure 4.

Using CID-MS^2 (A), ETD-MS^2 (B), and CID-MS^3 (C) to analyze a disulfide scrambled peptide (anti-HER2).

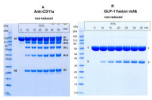

Figure 5.
Heat-stressed study for anti-CD11a (A), and GLP-1 with IgG4-Fc fusion mAb (B) using SDS-PAGE separation without DTT reduction.

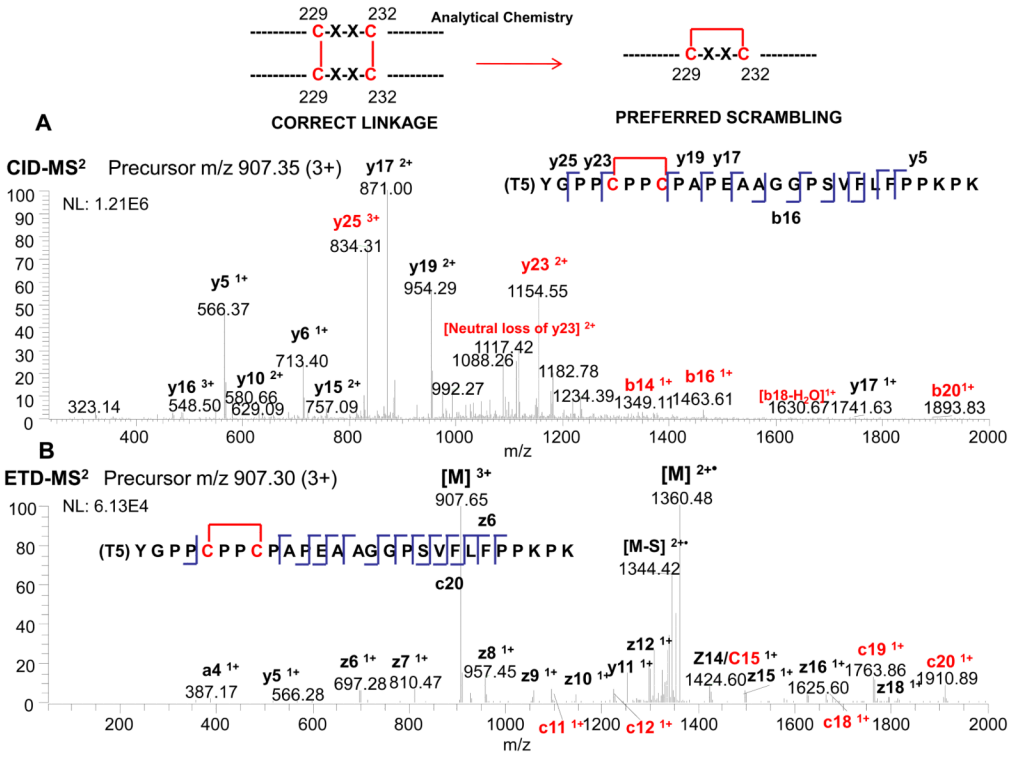

Figure 6.

CID and ETD analysis of an inter- to intra-chain disulfide scrambled peptide in GLP-1 with IgG4-Fc fusion mAb. The fragment ions containing the intra-chain disulfide were labeled with red, otherwise with black color.

Table 1

Theoretical tryptic peptides of anti-HER2 mAb without reduction, including linked disulfides (lines), cysteine position (parentheses), and molecular weight.

Tryptic peptides in heavy chain			
T2	20-30	LSCAASGFNIK **(Cys 22)**	MW= 2384.0934
T11	88-98	AEDTAVYYCSR **(Cys 96)**	
T14	137-150	STSGGTAALGCLVK **(Cys 147)**	MW= 7916.9351
T15	151-213	DYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVV TVPSSSLGTQTYICNVNHKPSNTK **(Cys 203)**	
T19	226-251	THTCPPCPAPELLGGPSVFLFPPKPK **(Cys 229&Cys 232)**	MW= 5454.8148
T19	226-251	THTCPPCPAPELLGGPSVFLFPPKPK **(Cys 229&Cys 232)**	
T21	259-277	TPEVTCVVVDVSHEDPEVK **(Cys 264)**	MW= 2328.1135
T27	324-325	CK **(Cys 324)**	
T35	364-373	NQVSLTCLVK **(Cys 370)**	MW= 3844.8393
T40	420-442	WQQGNVFSCSVMHEALHNHYTQK **(Cys 428)**	
Tryptic peptides in light chain			
T2	19-24	VTITCR **(Cys 23)**	MW= 4819.2579
T7	67-103	SGTDFTLTISSLQPEDFATYYCQQHYTTPPTFGQGTK **(Cys 88)**	
T10	127-142	SGTASVVCLLNNFYPR **(Cys 134)**	MW= 3555.7647
T17	191-207	VYACEVTHQGLSSPVTK**(Cys 194)**	
Tryptic peptide between heavy and light chain			
T18 (H)	222-225	SCDK **(Cys 223 from H chain)**	MW= 912.3587
T19 (L)	212-214	RGEC **(Cys 214 from L chain)**	
Lys-C digested peptide between heavy and light chain			
T18 (H)	222-225	SCDK **(Cys 223 from H chain)**	MW= 1260.5021
T18T19 (L)	208-214	SFNRGEC **(Cys 214 from L chain)**	

Table 2

The assignment of all correct disulfides in band A1 from the time point at 0 min of Figure 3A with the observed m/z at the charge of the highest intensity and the retention time.

	Observed m/z (charge)	Highest intensity	Retention time (min)
Correct disulfide in heavy chain			
T2 with T11	796.0530 (3+)	1.38E+07	26.50
T14 with T15	1132.7753 (7+)	2.73E+06	37.47
T19 with T19	780.7271 (7+)	1.11E+05	35.76
T21with T27	777.3900 (3+)	2.25E+07	25.72
T35 with T40	770.3930 (5+)	1.07E+07	29.05
Correct disulfide between heavy and light chains			
T18 (H) with T18T19 (L)*	631.3666 (2+)	6.13E+05	23.83
Correct disulfide in light chain			
T2 with T7	965.4877 (5+)	9.76E+06	34.11
T10 with T17	712.5760 (5+)	2.38E+07	31.97

* obtained from Lys-C digestion.

Table 3

The assignment of different chain combinations along with their corresponding disulfide linkages and major scrambled disulfides in the gel bands from the time point at 35 min of Figure 3A.

Band #	Chain combination[a]	Disulfides	Observed m/z (charge)	Highest intensity	Retention time (min)
A1	2 heavy chains + 2 light chains (144 kDa)	T18 (H) with T18T19 (L)[b]	631.3658 (2+)	2.09E+05	23.49
		T19 (H) with T19 (H)	780.7271 (7+)	6.11E+04	35.76
A1a	2 heavy chains + 1 light chain (121 kDa)	T2 (H) with T21 (H)	798.4250 (4+)	8.35E+05	29.74
		T18T19 (L)[b] with T18T19 (H)[c]	795.6248 (5+)	2.76E+04	32.46
A1b	2 heavy chains (98 kDa)	T11 (H) with T18T19 (H)[c]	740.5518 (6+)	1.71E+05	32.26
		T11 (H) with T18T19 (H)[c]	740.5517 (6+)	4.10E+05	32.6
A1c	1 heavy chain + 1 light chain (72 kDa)	T18T19 (H)[c] with T18T19 (H)[c]	791.4333 (8+)	1.41E+06	36.24
		T18T19 (L)[b] with T18T19 (H)[c]	795.4141 (5+)	5.71E+05	32.74
		Intra-chain disulfide in T19 (H)	683.1274 (4+)	8.37E+05	34.75
A1d	1 heavy chain (49 kDa)	T11 (H) with T35 (H)	595.8024 (4+)	2.38E+06	27.58
		T11 (H) with T18T19 (H)[c]	740.5486 (6+)	2.27E+06	32.28
		Intra-chain disulfide in T19 (H)	683.1265 (4+)	5.55E+06	34.28
A2	1 light chain (23 kDa)	Intra-chain disulfide in T19 (H)	683.1304 (4+)	6.64E+05	34.35
		T2 (L) with T17 (L)	628.0838 (4+)	3.18E+06	25.85

[a] The assignments were based on the disulfides and peptide sequences found at their corresponding gel bands.

[b] not cleaved (miscleavage) between T18 and T19 of the light chain.

[c] not cleaved (miscleavage) between T18 and T19 of the heavy chain; in which T19 formed an intra-chain disulfide.