In vitro and in vivo modifications of recombinant and human IgG antibodies

Hongcheng Liu1,*, Gomathinayagam Ponniah1, Hui-Min Zhang2, Christine Nowak1, Alyssa Neill1, Nidia Gonzalez-Lopez1, Rekha Patel1, Guilong Cheng1, Adriana Z Kita1, and Bruce Andrien1

1Protein Characterization; Alexion Pharmaceuticals Inc.; Cheshire, CT USA; 2Bioprocess Development; Merck Research Laboratories; Merck; Kenilworth, NJ USA

Keywords: recombinant monoclonal antibodies, endogenous IgG antibodies, posttranslational modifications, pyroglutamate, leader sequence, C-terminal lysine, oligosaccharides

Tremendous knowledge has been gained in the understanding of various modifications of IgG antibodies, driven mainly by the fact that antibodies are one of the most important groups of therapeutic molecules and because of the development of advanced analytical techniques. Recombinant monoclonal antibody (mAb) therapeutics expressed in mammalian cell lines and endogenous IgG molecules secreted by B cells in the human body share some modifications, but each have some unique modifications. Modifications that are common to recombinant mAb and endogenous IgG molecules are considered to pose a lower risk of immunogenicity. On the other hand, modifications that are unique to recombinant mAbs could potentially pose higher risk. The focus of this review is the comparison of frequently observed modifications of recombinant monoclonal antibodies to those of endogenous IgG molecules.

Introduction

Most recombinant monoclonal antibody (mAb) therapeutics are produced in one of three mammalian cell lines, Chinese hamster ovary (CHO), murine NS0 or murine SP2/0. Although, in general, the amino acid sequence of recombinant mAbs are expressed in those cell lines with high fidelity, low levels of variation have been observed. The use of non-human cell lines can introduce post-translational modifications that are not intrinsically present in the human body. Such unnatural modifications may also be introduced during the period between purification and patient administration. The presence of those modifications is a concern due to the possibility of undesired effects such as loss of efficacy and increased immunogenicity.

In this review, we compiled data on modifications that occur in recombinant mAbs with the aim of answering three questions: 1) What modifications occur?; 2) What happens to recombinant monoclonal antibodies with those modifications in vivo?; and 3) Are the same modifications present in endogenous human IgGs? An underlying assumption is that a particular modification should pose a lower risk if it can be removed rapidly in circulation or if it is also present in endogenous IgG. The main categories discussed here are N-terminal modifications, C-terminal modifications, oligosaccharides, degradation of asparagine and aspartate, oxidation of methionine and tryptophan, cysteine-related variants and glycation. For each category, specific modifications will be discussed first for recombinant mAbs and then endogenous IgG antibodies.

N-Terminal Modifications

Cyclization of the N-terminal glutamine (Gln) or glutamate (Glu) to form pyroglutamate (pyroE) and incomplete removal of leader sequence are the two major types of N-terminal modifications. Truncation of the N-terminus resulting in the light chain lacking two amino acids has been reported in a recombinant mAb.1 So far, however, truncation has not been established as a general modification of recombinant mAbs.

N-Terminal Pyroglutamate

It is common that the first amino acid of the light chain, heavy chain or both is either Gln or Glu, encoded in the genes. Spontaneous cyclization of N-terminal Gln2–4 and to a lesser degree, N-terminal Glu5–7 results in the formation of pyroE. The presence of pyroE has no effect on antibody structure8 and antigen binding.8 In addition, no difference in in vivo clearance between antibodies with N-terminal Glu compared with antibody with N-terminal pyroE has been observed.9 One study demonstrated that the levels of pyroE of a recombinant mAb recovered from rat serum after 1 h in circulation did not show much difference compared with the starting material.10 However, the reaction of cyclization of Gln is expected to continue in circulation because of the non-enzymatic nature of the reaction. Using a synthesized peptide, it was found that Gln was converted to pyroE at a rate of 1.41% per hour in cell culture.8 Assuming a comparable in vivo rate, conversion of Gln to pyroE will be complete within a day because the majority of the N-terminal Gln of most recombinant mAbs is already cyclized after purification. The conversion from Glu to pyroE of recombinant mAbs
continues in vivo and pyroE naturally exists in endogenous human IgG. Overall, this type of N-terminal modification is not expected to have a substantial effect on efficacy and safety.

**Partial Leader Sequence**

Incomplete removal of leader sequence has also been observed for recombinant mAbs. Typically, only a portion of the leader sequence remains attached to the antibody instead of the entire leader sequence. The presence of a portion of the leader sequence has no effect on antigen binding, structure, FcRn binding, or pharmacokinetics. Signal peptides are composed of a hydrophobic region that is flanked by a polar region often with net positive charge on the N-terminal side and a polar region containing proline (Pro) and glycine (Gly) with small uncharged residues at positions -3 and -1 on the C-terminal side. It is unlikely that the remaining leader sequence of recombinant mAbs will be removed in circulation because the remaining portion of the leader sequence does not have the structural characteristics required for cleavage. The presence of partial leader sequence in recombinant mAbs may likely be due to malfunctions of the cell machinery of the recombinant cell lines that are under stress to produce extremely high levels of proteins. In this sense, endogenous IgG antibodies should not have a partial leader sequence under normal physiological conditions. However, the presence of a partial leader sequence may not be a concern if human leader sequences were used for making the constructs of recombinant mAbs.

**C-Terminal Modifications**

The heavy chain C-terminal amino acid sequences encoded in the genes are PGK for IgG1, IgG2, IgG3, and LGK for IgG4. The first major modification is the removal of C-terminal lysine (Lys). The second major modification is amidation of C-terminal Pro residue was first identified in recombinant monoclonal IgG1 antibody. Later, another study demonstrated amidation is probably a common modification of mAbs because it was observed in multiple IgG1 and IgG4 molecules. Amidation of IgG2 and IgG3 is also expected because IgG1, IgG2 and IgG3 share the same C-terminal sequence. The level of Pro amidation increased with the increase of copper added to the culture medium. Amidation has no effect on antigen binding and Fc effector functions. Amidation was not detected in endogenous human IgG antibodies. However, because amidation has been commonly observed in biologically active peptides including peptide hormones and neurotransmitters in humans, it is not considered an unnatural modification to the human immune system.

**Oligosaccharides**

Oligosaccharides are a well-studied modification of antibodies. In addition to glycosylation of the conserved asparagine (Asn) in the CH2 domain, 20–30% of human IgGs include N-linked glycosylation in variable domains. N-linked glycosylation in the variable domains has been reported for recombinant mAbs. Atypical glycosylation of recombinant mAbs has also been reported, including O-fucosylation of a serine (Ser) residue in light chain complementary-determining region (CDR) and N-glycosylation of Asn and Gln residues in non-consensus sequences. These atypical glycosylations are only present to an extreme low level. A single fucosylation has been reported in human urinary-type plasminogen activator and N-glycosylation of Asn in non-consensus sequence has also been observed in human endogenous IgG. Therefore, those modifications may not be a concern with regard to immunogenicity.

The major glycoforms of recombinant mAbs expressed in CHO, murine NSO, and murine SP2/0 cell lines are G0F, G1F and G2F. Some minor species are also common, including low percentages of afucosylated complex, high mannosylated, and hybrid oligosaccharides and low percentage of aglycosylated species. The major difference between CHO cell lines and the two mouse cell lines is the presence of immunogenetic α1, 3 gal and N-glycolyneuraminic acid (Neu5Gc) in recombinant mAbs expressed using murine cell lines. The presence of oligosaccharides is critical for the structural integrity, stability and functions of IgG molecules, as will be discussed later, but specific structures are also important. For example, lack of core-fucose results in IgG molecules with higher affinity to FcyIII receptor and enhanced ADCC while the presence of a terminal galactose or bisecting residue only has a subtle effect on receptor binding and ADCC. Human IgG antibodies share the same major and minor glycoforms with recombinant mAbs.

The majority of the oligosaccharides of human and recombinant IgGs include core-fucose. In most cases, the levels of...
Aglycosylated Antibodies

Compared with glycosylated antibodies, antibodies without oligosaccharides show conformational changes, decreased stability, increased propensity to aggregate, and almost complete loss of effector functions. The effect of aglycosylation on antibody half-life cannot be generalized because there are studies demonstrating shorter half-life and normal half-life. A very low level (0.1%) of aglycosylation has been found in human IgG. Clinical experience with aglycosylated antibodies did not demonstrate increased risk.

High Mannose

High mannose oligosaccharides have been commonly observed in recombinant mAbs at higher levels than in endogenous IgG antibodies. Antibodies with high mannose showed defects in Fc effector functions. Although some studies showed no difference in clearance, the majority showed a faster clearance of antibodies with high mannose. High mannose with greater than five mannose residues can be converted to mannose 5 because of mannosidase activity in circulation. High mannose has been observed in endogenous human IgG at a very low level.

Immunogenic Oligosaccharides

α-1,3 gal is not present in endogenous human IgG antibodies due to the absence of the gene for the synthesizing enzyme, α-1,3-galactosyltransferase. Therefore, α1,3 gal is foreign to the human immune system. The presence of IgE antibodies specific to α-1,3 gal in the Fab region of cetuximab has been reported to cause hypersensitivity in some patients. Neu5Gc is not normally present in human IgG because of the lack of CMP-N-acetylneuraminic acid hydroxylase activity due to mutation in the gene. However, Neu5Gc can be metabolically incorporated into human cells because of diet or cell culture medium containing animal derived material. Anti-Neu5Gc antibodies have been detected or induced because of exposure to Neu5Gc in humans.

Degradation of Asparagine and Aspartate

Deamidation is the major degradation pathway of Asn, which results in the formation of aspartate (Asp) and isoaspartate (IsoAsp). IsoAsp can also be formed from isomerization of Asp, which is another major degradation pathway of recombinant mAbs. Deamidation and isomerization share the same reaction intermediate, succinimide. Deamidation of recombinant mAbs and endogenous IgG antibodies can occur in vivo in monkeys as well as in humans. Deamidation and isomerization has been implicated in aging and several age-related diseases, and the existence of the repairing enzyme, protein isoaspartate methyl transferase, further highlights the importance of deamidation and isomerization in vivo.

Deamidation

Deamidation of Asn has been widely reported in recombinant mAbs in either the CDR regions or in the constant regions. Increased thermal stability of Fab with Asp and decreased thermal stability of Fab with isoAsp compared with Fab with the original Asn residue were observed for a recombinant mAb as a result of deamidation. Several studies have demonstrated that deamidation in the CDR regions resulted in decreased binding affinity and potency. As expected, deamidation in the constant domain has no effect on antigen binding. Deamidation of Asn residues in CDR regions continues in vivo in monkey serum. No preferential clearance is suggested by the constant ratio of Asp to isoAsp. Deamidation of Asn residues in the constant region of a recombinant mAb continues in circulation in human and deamidation of the same sites was also observed in endogenous IgG.

Isomerization

Isomerization of Asp to form isoAsp introduces a minimal charge difference. However, isomerization can cause a conformational change because of the introduction of an additional methyl group to the peptide backbone. An ~50% decrease in binding affinity was observed with one Fab with the original Asn and the other one with either isoAsp or succinimide. Isomerization of Asp102 in one of the heavy chain CDR3 resulted in an antibody only 9–21% as potent as the antibody with the original Asp. Isomerization of Asp92 in the light chain CDR3 of a recombinant monoclonal IgG2 antibody deactivated its antigen binding capability. As discussed in the previous section, isoAsp can be formed in vivo in monkey and in human in both recombinant mAbs and human endogenous IgG antibodies. Therefore, isoAsp from isomerization is not foreign to the human immune system, indicating a lower risk of immunogenicity.

Succinimide

Although succinimide is unstable, it has been detected in several recombinant mAbs. The presence of succinimide in the CDR regions of several antibodies from Asp isomerization resulted in decreased antigen binding and
Oxidation of Methionine, Tryptophan and Other Residues

Oxidation of recombinant mAbs has been commonly reported, mainly at methionine (Met) residues and less frequently at tryptophan (Trp), histidine (His) and other residues. Two conserved Met residues, Met252 and Met428, in the Fc region are highly susceptible to oxidation.\(^{87-92}\) Oxidation of Met residues in the Fc region has no effect on antigen binding,\(^{87}\) but results in a conformational change in the CH2 domain\(^{90,91}\) and decreased binding to protein A,\(^{89,93}\) protein G\(^{89}\) and FcRn.\(^{93-95}\) It also has a subtle effect on Fc receptors.\(^{94}\) Decreased half-life was only observed with relatively high levels of oxidation.\(^{95}\) Oxidation of Trp has only been reported in a few cases.\(^{92,96,97}\) In one of those studies, oxidation of the single Trp residue in the CDR3 caused a substantial decrease in antigen binding and potency.\(^{92}\) Oxidation of Trp to form various products during exposure to light or heat causes changes to the color of mAb products.\(^{98}\) Metal-catalyzed oxidation can also lead to oxidative carbonylation of Arg, Pro, Lys and Thr, especially when those residues are located on the surface of the molecule.\(^{99}\) The observation of oxidative carbonylation on mAbs in the unstressed drug substance indicates that such reactions can occur during manufacturing because of product contact with metal surface. Direct His oxidation\(^{100}\) and its further reaction product, His-His cross-linking,\(^{101}\) are observed when mAbs were exposed to light. Oxidation of Met and Trp are probably present in endogenous IgG from humans, especially for patients with inflammation.\(^{102,103}\) Oxidation of proteins including Met and Trp residues has been widely detected in vivo, and may result from aging and several pathological conditions.\(^{104}\)

Cysteine Related Variants

In the classical view, cysteine (Cys) residues are involved in formation of disulfide bonds with well-defined homogeneous linkage for each subclass of IgG antibodies. However, several variations have been discovered, including alternative disulfide bond linkage, trisulfide bond, thioether linkage, free Cys and racemization. Cysteinylination of Cys residues has also been observed, but only to antibodies with extra Cys residues,\(^{105}\) which is rare.

Alternative Disulfide Bond Linkage

Alternative disulfide bond linkage was first reported for IgG4. The two inter-heavy chain disulfide bonds of IgG4 exist in equilibrium with the formation of two intra-heavy chain disulfide bonds,\(^{106,107}\) which can result in the formation of half-molecules. The formation of half-molecules was almost eliminated when the Ser residue in the IgG4 hinge of CPSC was mutated to a Pro residue, thereby making an IgG1 hinge of CPCC.\(^{106-108}\) Trace amounts of half-molecule were also observed for IgG1.\(^{106}\) The other consequence of the instability of the IgG4 hinge is the formation of hybrid molecules between two different IgG4 molecules, which can occur in recombinant mAbs incubated with glutathione or injected into mice\(^{109}\) and naturally in humans.\(^{109,110}\)

In addition to the classical IgG2 disulfide bond structure, termed IgG2A, two additional structures, termed IgG2B and IgG2A/B were discovered.\(^{111}\) IgG2A has a larger hydrodynamic size than IgG2B,\(^{112}\) and, in a subset of IgG2, IgG2-A shows higher potency.\(^{112}\) Incubation of IgG2 in vitro with redox similar to human blood shows a decrease in IgG2A and an increase in IgG2B.\(^{112}\) A similar conversion from IgG2A to IgG2B was also observed in cell culture medium and in circulation after administration into human body.\(^{113}\) The exact isoforms are also naturally present in human IgG2.\(^{111,112}\)

Trisulfide Bonds

Trisulfide bonds were first reported in a recombinant monoclonal IgG2 antibody in the hinge region between the two heavy chains.\(^{114}\) It was later found that trisulfide bonds are present in all subclasses of recombinant IgGs.\(^{115}\) Higher percentages of trisulfide bonds were observed between light chain and heavy chain than between the two heavy chains and no trisulfide bonds were associated with intrachain disulfide bonds.\(^{115}\) The presence of trisulfide bonds has no effect on thermal stability,\(^{114}\) and antigen binding and potency.\(^{115,116}\) Trisulfide bonding has also been shown to affect the reduction step for the production of antibody-drug conjugation.\(^{117}\) Cell culture parameters such as scale and age have a significant effect on the level of trisulfide bonds.\(^{115,118}\) Trisulfide bonding can be eliminated by incubation with mild reducing reagents.\(^{114,115}\) Trisulfide bonds were stable in vitro in buffers and in rat serum; however, they are completely converted to disulfide bonds after 24 h in vivo in rat serum.\(^{115}\) Trisulfide bonds between the light chain and heavy chain were also found in endogenous IgG.\(^{115,119}\)

Thioether

Thioethers between the light chain and heavy chain were first identified in a recombinant monoclonal IgG1 antibody.\(^{120}\) Higher pH promotes the formation of a thioether bond.\(^{121}\) A thioether between the light chain and heavy chain increases at about 0.1%/day for therapeutic antibodies in healthy volunteers.\(^{119}\) There is no clearance difference between antibodies with and without a thioether.\(^{119}\) Thioethers naturally exist in human endogenous IgG molecules, 11.0% for IgG1, and about 5.2% for IgGk.\(^{119}\)
Free Cysteine

Low levels of free Cys have been widely observed for recombinant mAbs, especially under denaturing conditions in the range from trace level to 2.5 moles per mole of IgG.10,122,125 Free sulfhydryl is associated with every single cysteine residue in the IgG molecules.126,127 In some cases, antibodies with incomplete formation of the intrachain disulfide bond in the heavy chain variable domain were identified as separated peaks.10,122,126 Higher levels free sulfhydryl resulted in decreased thermal stability,124 formation of covalent aggregates123 and decreased potency.122 One study showed a slightly higher antigen binding with no difference in complement-dependent cytotoxicity.128 Intrachain disulfide bond in the heavy chain variable domain can be rapidly formed from the free sulfhydryl state during in vitro incubation with 5,5'-dithiobis-(2-nitrobenzoic acid), in rat serum and human serum or after circulation in rat serum in vivo.10 Free cysteine has also been detected in human endogenous IgG antibodies with levels varied in different studies,123,124,129,130 probably due to difference in methods.

Racemization

Racemization of the heavy chain Cys residue involved in the formation of the inter-heavy/light chain disulfide bond from L-Cys to D-Cys was observed in a recombinant monoclonal IgG1 antibody during storage.131 It was later found that all Cys residues involved in interchain disulfide bonds of IgG1 and IgG2 can be racemized to D form to some degree under basic conditions.132 In addition, racemization was also observed in endogenous IgG molecules from human.132

Glycation

Glycation is a non-enzymatic reaction between reducing sugars and protein N-terminal amino group or the side chain of Lys residues. Glycation occurs during cell culture, formulation and storage,133,134 where reducing sugars are used or generated from non-reducing sugars. Glycation susceptibility is altered by the surface accessibility of the Lys residues,135 and can also be catalyzed by amino acids in close proximity.136 Glycation can be controlled by optimizing cell culture conditions.141 A mAb containing 17% glycation did not show any structural difference compared with the main peak with no glycation.11 However, glycation increases the propensity of aggregation of recombinant mAbs.143 Several studies demonstrated glycation of Lys in various CDR regions from 10% glycation to about 100% had no effect on antigen binding and potency.11,138,140 Extensive glycation has no effect on binding to FcγRIIIa and FcγRn and protein A.144 However, those results can only demonstrate that those Lys residues are not critical for various ligand binding. A significant effect is expected if the glycated residues are localized in the binding pockets because of the loss of the positive charge of Lys upon glycation. Acidic species with 17% glycation did not show difference in pharmacokinetics in rats.11 Glycation of recombinant mAbs increases with the increase of circulation time in human.144 Glycation has also been detected in endogenous IgG of healthy subjects with a comparable rate as recombinant mAb in circulation.144 The risk of glycation of recombinant mAbs may be low due to its low level and its presence in vivo. However, it is worthwhile to mention that antibodies targeting glycated IgG has been observed in rheumatoid arthritis patients145 and the interaction of advanced glycation end product (AGEs) with AGE-specific receptors can stimulate the generation of reactive oxygen species and inflammation.146

Low Level of Sequence Variant

A low level of tyrosine (Tyr) to Gln sequence variation that occurred during transfection of a recombinant mAb expressed in CHO was first reported in 1993.147 Low levels of sequence variants have been more widely reported recently,148-154 which can be attributed to the advance of modern analytical techniques offering much higher sensitivity. Sequence variants are introduced because of mutation at the DNA level,153,155 during transfection,147 or translation.148,154,155 Sequence variants of recombinant mAbs can be eliminated depending on the specific causes. For example, codon-specific low levels of Ser replaced by Asn can be eliminated by changing the codon from AGC to another Ser codon.155 Misincorporation of amino acids due to amino acid starvation can be eliminated by providing sufficient amounts of the specific amino acids.149,150,154 It should be mentioned that low levels of misincorporation can occur naturally,156 and it should not be a surprise that endogenous IgG molecules also contain low levels of sequence variants. Low levels of sequence variation may never be completely eliminated. In this case, maintaining misincorporation at an extremely low level and ensuring batch-to-batch consistency may be more practical and important for the production of recombinant mAbs.

Others

Several other modifications have been reported, but only in limited cases. Expression of intron sequence results in a recombinant mAb with an additional 24 amino acids between the variable and constant domain of the heavy chain.3 Homologous recombination between the light chain gene and the heavy chain gene results in an antibody with a minor species of heavy chain containing the light chain variable domain.157 Reaction of methylglyoxal, a by-product of the tricarboxylic acid cycle, with arginine can result in increased levels of acidic species of mAb.158

Modifications and Their Importance

A thorough characterization of mAbs and their major degradation pathways is required for development of mAb therapeutics. Such efforts have provided in-depth understanding of the
The major known modifications in recombinant mAbs and their presence in endogenous IgG molecules are summarized in Table 1. We defined the importance of each modification based on an overall evaluation of their prevalence, importance to safety, immunogenicity, and efficacy. For example, pyroE is widely observed, but it is not important because it has no effect on structure, stability, and function and it is endogenous. On the other hand, degradation of Asn and Asp is considered to be important because it has been widely observed and can affect mAb structure, stability, and functions. In addition, although degradation of Asn and Asp has been observed in endogenous IgG molecules, exposure of patients to recombinant mAbs with high levels of those degradation products may still pose a high risk. Although sharing some common features, each mAb is different and should be evaluated on a case-by-case basis. Modifications that are not defined as highly important should also be controlled. Consistency of various modifications from batch to batch is necessary to demonstrate a well-controlled process.

### Conclusions

The development of recombinant monoclonal mAbs has evolved from mouse, chimeric, humanized to human antibodies with the goal of reducing immunogenicity. Although significant progress has been made to achieve this goal, significant immunogenicity risks remain even for fully human antibodies. Therefore, the identification and mitigation of non-human modifications of recombinant monoclonal therapeutics is crucial.

By far, the host cell line plays the most important role in introducing non-human modifications. For example, α-1,3-gal and Neu5Gc are the natural products of using murine cell lines, where optimizing cell culture conditions may not be sufficient to eliminate those modifications. However, cell culture conditions could have a bigger effect on several other modifications, such as N-terminal cyclization, C-terminal Lys removal, glycation, trisulfi de bonds, and IgG2 isoforms. Various modifications introduced during purification and storage, such as glycation, deamidation, N-terminal cyclization, can also be controlled by lowering temperature and optimizing pH and excipients. Some of the modifications may be eliminated rapidly in circulation, thus lowering the risk of immunogenicity. In general, the human body can better tolerate modifications that are natural compared with those that are not.

Thorough characterization of modifications and risk assessment at an early stage of development based on the understanding of the nature of modifications and the production of batches consistent in quality attributes throughout clinical stage and commercial stage are critical to ensure the supply of efficacious and safe products.

### Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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**Table 1. Common modifications of recombinant mAbs, and their presence in endogenous IgG and importance**

| Modifications                             | Endogenous IgG | Importance |
|-------------------------------------------|----------------|------------|
| N-terminal modifications                  |                |            |
| Truncation                                | ?              | +          |
| PyroE                                     | Yes            | +          |
| Partial leader sequence                   | Yes            | +          |
| C-terminal modifications                  |                |            |
| C-terminal Lys                            | Yes            | +          |
| Amidation                                 | No             | +          |
| Oligosaccharides                          |                |            |
| Aglycosylated species                     | Yes            | +          |
| High mannose                              | Yes            | +          |
| Neu5Gc                                    | No             | +++        |
| α1,3-gal                                  | No             | +++        |
| Degradation of Asn and Asp                |                |            |
| Asn deamidation                           | Yes            | +++        |
| Isomerization                             | Yes            | +++        |
| Succinimide                               | Yes            | +++        |
| Oxidation                                 |                |            |
| Met oxidation                             | Yes            | ++         |
| Trp oxidation                             | Yes            | ++         |
| His oxidation and cross-link              | ?              | +          |
| Cysteine-related                          |                |            |
| Alternative disulfide bond linkage        | Yes            | ++         |
| Trisulfide bond                           | Yes            | ++         |
| Thioether                                 | Yes            | ++         |
| Free cysteine                             | Yes            | ++         |
| Racemization                              | Yes            | ++         |
| Cystenylation                             | ?              | +          |
| Glycation                                 | Yes            | ++         |
| Low level sequence variants               | ?              | +          |
| Others                                    |                |            |
| Intron sequence                           | ?              | +          |
| Methyglyoxal modification                 | ?              | +          |

+++ ++ indicates the highest level of importance, + indicates the lowest level of importance.

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