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Development of a sample preparation method for monitoring correct disulfide linkages of monoclonal antibodies by liquid chromatography–mass spectrometry

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ABSTRACT

The number and positions of disulfide linkages in a therapeutic monoclonal antibody (mAb) play a crucial role in forming and stabilizing a correct mAb structure that is critical to its function. Peptide mapping by liquid chromatography–mass spectrometry (LC–MS) analysis of enzymatically digested mAb under nonreducing condition is a powerful method for disulfide linkage characterization to ensure mAb drug function and quality. However, the development of a robust sample preparation method with improved digestion efficiency and minimized disulfide scrambling for disulfide linkage analysis is essential but challenging. In this study, a sample preparation method for analysis of correct disulfide linkages in therapeutic mAbs was developed. Instead of common trypsin digestion, Lys-C plus trypsin was used in this approach to improve digestion efficiency. In addition, lower digestion temperature (25 °C) and lower digestion pH (pH 6.8) were also examined to minimize disulfide scrambling. Our results showed that Lys-C plus trypsin digestion at pH 6.8 and 25 °C is a better sample preparation condition for all therapeutic mAbs tested in this study because of a better digestion efficiency (all expected disulfide linkages can be confidently observed) and minimal disulfide scrambling.

Therapeutic monoclonal antibodies (mAbs) are powerful for treatment of many types of diseases due to their high specificities and fewer side effects [1]. Currently, all therapeutic mAbs on the market are immunoglobulin G (IgG)-type mAbs. IgG is composed of two identical heavy chains (HCs) and two identical light chains (LCs) linked by disulfide bonds to form a "Y"-shaped structure (Fig. 1). Each chain is composed of domains, and the domains are held together by an intra-chain disulfide bond. The light chain has one variable domain (VL) and one constant domain (CL). The heavy chain consists of one variable domain (VH) and three constant domains (CH1, CH2, and CH3). In addition, one inter-chain disulfide bond links the light chain and heavy chain together, and two or more inter-chain disulfide bonds in the hinge region connect two heavy chains together. IgGs can be further divided into four subclasses, and they are named in order of their abundances in circulation: IgG1, IgG2, IgG3, and IgG4. In addition to amino acid differences, the disulfide bonds, especially hinge region disulfide bonds and the inter-chain disulfide bond between heavy and light chains, are different among IgG subclasses and even IgG2 subisotypes (Fig. 1) [2].

The number and positions of disulfide linkages are critical to their biological function and stability. For example, IgG3 has 11 inter-heavy chain disulfide bonds within its long hinge region. The longest hinge of IgG3 makes it the most flexible, and therefore the most effective, complement activator among all IgGs [3]. By contrast, IgG4 does not activate complement due to steric hindrance of the complement binding sites caused by its structure [3]. However, the long hinge region of IgG3 is prone to proteolysis and results in a short half-life *in vivo* (~7 days vs. 21 days for IgG1, IgG2, and IgG4) [4,5]. Due to its short half-life, IgG3 is not commonly used as a therapeutic agent, whereas IgG1, IgG2, and IgG4 types of mAbs are widely used for therapeutic purposes.

Abbreviations used: mAb, monoclonal antibody; IgG, immunoglobulin G; HC, heavy chain; LC, light chain; VL, variable domain of light chain; CL, constant domain of light chain; VH, variable domain of heavy chain; CH, constant domain of heavy chain; LC–MS, liquid chromatography–mass spectrometry; NEM, *N*-ethylmaleimide; MS, mass spectrometry; ACN, acetonitrile; TFA, trifluoroacetic acid; DTT, dithiothreitol; IAM, iodoacetamide; CHO, Chinese hamster ovary; EIC, extracted ion chromatogram; RSD, relative standard deviation.

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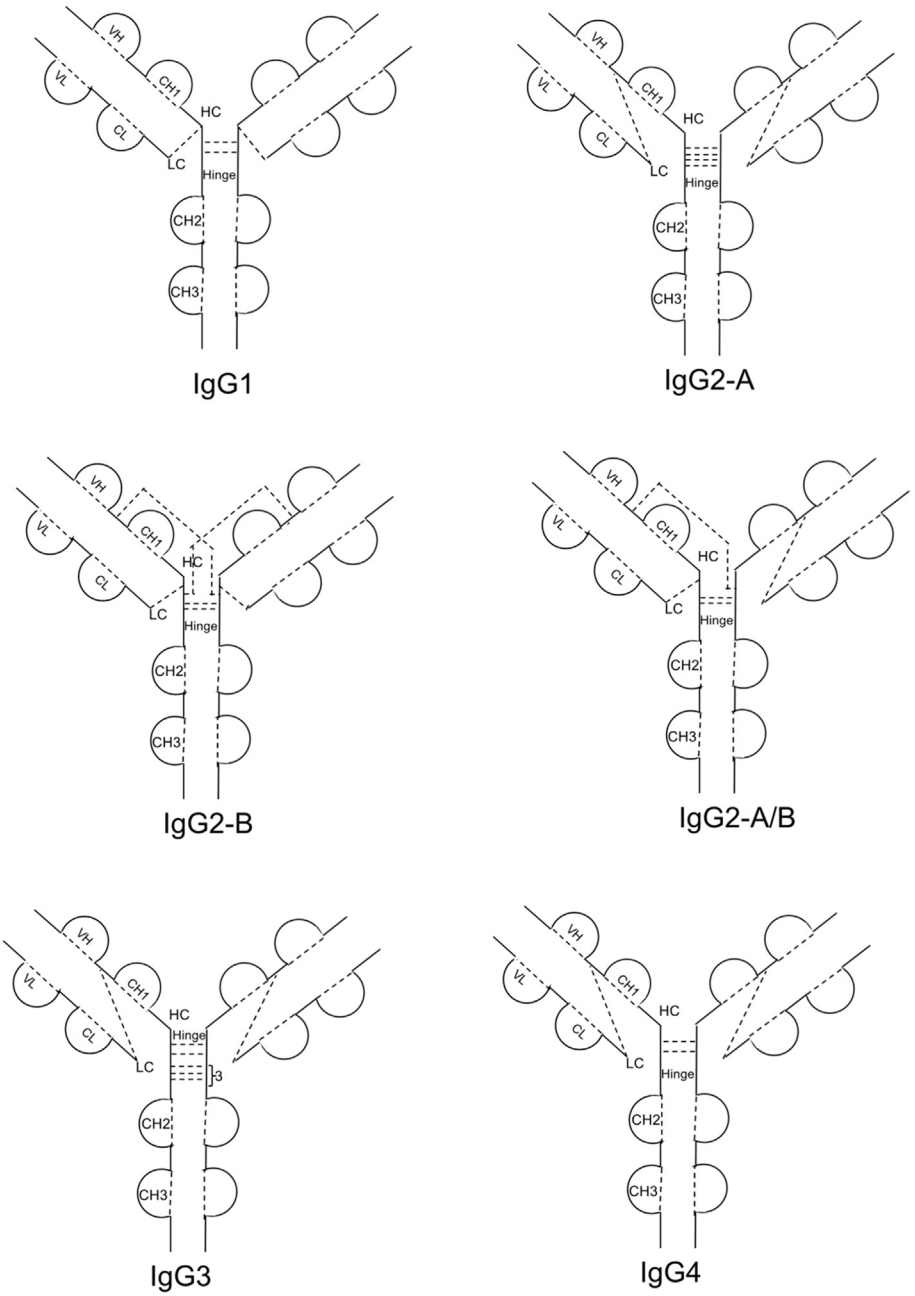

Fig.1. Schematic structure of IgG antibodies. The dashed lines represent disulfide linkages.

It is important to characterize the disulfide linkages to ensure mAb drug function and quality. Peptide mapping by liquid chromatography–mass spectrometry (LC–MS) analysis of enzymatically digested mAb under nonreducing and reducing conditions is the method often used for disulfide linkage assignment [6]. By comparison of the peaks from these two conditions, a disulfide linkage can be identified by comparing the nonreducing and reducing peptide mapping [6]. Alternatively, the disulfide linkage can be directly characterized by electron transfer dissociation (ETD) and collision-induced dissociation (CID) fragmentation of non-reduced peptides [7].

However, all of these approaches require a robust sample preparation method with high digestion efficiency on correct disulfide-linked peptides and minimal disulfide scrambling. If relative quantitation needs to be performed, good repeatability of the sample preparation is necessary. The development of such a method is challenging. First, free cysteine residues have been detected in all types of IgG antibodies, and the free cysteine residues can easily act with each other to form scrambled disulfide bonds at basic pH [8]. Furthermore, the correct disulfide bonds can break up due to β-elimination at elevated temperature under basic pH to form scrambled disulfide bonds or a thioether [8]. However, the commonly used enzymes, such as trypsin and Lys-C, are mostly active at 37 °C and pH 8.0. These conditions, therefore, will facilitate the formation of artificial disulfide linkages. In addition, enzymatic digestion is often less effective for the peptides containing disulfide linkages due to the steric hindrance [9].

Because all types of IgGs have a similar Y-shaped structure, a sample preparation method for analysis of all correct disulfide linkages in three types of IgG mAbs (IgG1, IgG2, and IgG4) with