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Application of Dual Protease Column for HDX-MS Analysis of Monoclonal Antibodies

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ABSTRACT

A co-immobilized, dual protease column was developed and implemented to more efficiently digest IgG molecules for hydrogen/deuterium exchange mass spectrometry (HDX-MS). The low-pH proteolytic enzymes pepsin and type XIII protease from *Aspergillus* were packed into a single column to most effectively combine the complementary specificities. The method was optimized using an IgG2 monoclonal antibody as a substrate because they are known to be more difficult to efficiently digest. The general applicability of the method was then demonstrated using IgG1 and IgG4 mAbs. The dual protease column and optimized method yielded improved digestion efficiency, as measured by the increased number of smaller, overlapping peptides in comparison with pepsin or type XIII alone, making HDX-MS more suitable for measuring deuterium uptake with higher resolution. The enhanced digestion efficiency and increased sequence coverage enables the routine application of HDX-MS to all therapeutic IgG molecules for investigations of higher order structure, especially when posttranslational and storage-induced modifications are detected, providing further product understanding for structure–function relationships and ultimately ensuring clinical safety and efficacy.

Introduction

Monoclonal antibodies (mAbs) are an important class of biotherapeutic molecules. There are ~40 mAbs or mAb-like molecules approved as therapies for multiple diseases, such as rheumatoid arthritis and other inflammatory diseases, and several types of cancers.[1] Successful clinical development and commercialization of a therapeutic antibody requires tight control of product quality and batch-to-batch consistency to ensure sustained safety and efficacy.

A battery of quality control assays, including a functional assessment, is performed on every manufactured batch for release and therapeutic use. In contrast, in-depth, heightened product characterization is carried out at specific stages of product development on representative batches in an effort to build product and process knowledge. Both quality control release assays and heightened characterization with state-of-the-art instruments and methods are crucial for establishing a reference material (RM). A well-characterized RM essentially embodies process and product knowledge and becomes a vital part of future product comparability assessments where several batches produced before and after a manufacturing process or site and formulation change are closely evaluated against each other and RM in side-by-side experiments.[2,3]

Heightened product characterization involves a systematic evaluation of the primary structure, posttranslational modifications, and higher order structure (HOS) to ensure the therapeutic IgG antibody has the expected amino acid sequence and disulfide bonding,[4] predicted N- and C-terminal heterogeneity, typical N-linked glycosylation profile,[5] negligible chemical modifications, expected secondary and tertiary structural features, and reasonable overall thermal stability. Although ultra–high-resolution mass spectrometry (MS) in combination with ultra–high-performance liquid chromatography (UHPLC) and modern column technologies provide unequivocal comparative data for the product quality attributes pertaining to primary structure and posttranslational modifications, the mainstream spectroscopic and calorimetric techniques for HOS comparability assessments have less discriminating power for absolute structural assignments, thereby encouraging development of next-generation HOS techniques and methods.

Current methods for HOS determination may include techniques such as circular dichroism spectroscopy, tryptophan emission fluorescence spectroscopy, differential scanning calorimetry, ultraviolet absorption spectroscopy, Fourier transform infrared

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spectroscopy, analytical ultracentrifugation, and light scattering methods.[6,7] These methods are useful for obtaining data on differences in overall secondary, tertiary, and quaternary structure in a comparative mode but lack sufficient resolution to determine the specific domains or sites contributing to any differences observed.[8] Hydrogen–deuterium exchange mass spectrometry (HDX-MS) offers potential to not only perform comparisons of overall HOS differences but also yields information on the sites differing in conformation.[9-11] In this method, the level of deuterium uptake is determined at various time points by measuring the increase in mass.[12,13] Amide hydrogens in the polypeptide backbone exchange with solvent on a timescale amenable to measurement using mass spectrometry.[14] Flexible, solvent-accessible regions exchange amide hydrogens more rapidly than regions that are not accessible to solvent or that are involved in hydrogen bonding.

Typically in HDX-MS, deuterated protein samples are subjected to pepsin digestion to generate proteolytic peptides for localizing potential HOS differences. In a comparison of a control (i.e., RM) versus an experimental sample, differences in deuterium uptake and the sites of these differences can be determined by measuring the extent of mass increase for each respective proteolytic peptide in the 2 proteins.[15] HDX-MS is useful for many aspects of biotherapeutics development[16,17] including ligand-binding studies,[18-20] epitope mapping,[21,22] in-process chemical modifications,[23] effect of excipients,[24] antibody–drug conjugate structural studies,[25,26] and biosimilarity assessments.[27]

To achieve useful information from HDX-MS experiments, the digestion method needs to be optimized for the particular class of molecule. The method used should not only obtain maximum sequence coverage but should also yield peptides of optimal size for effectively measuring deuterium uptake. Additionally, the method should yield overlapping peptides along the protein backbone to improve the resolution of sites showing any differences and generate peptide redundancy at as many sites as possible to improve the confidence in the measurement of any potential differences. The efficiency of the proteolytic digestion is critical for obtaining this additional layer of peptide information, but it needs to be balanced by the need to minimize back exchange, which can confound the experiment. For HOS analysis of mAbs using HDX-MS, this is made difficult by the large molecular size and general resistance of IgG2 mAbs to protease digestion, particularly in the hinge region.[28] All approved therapeutic mAbs are IgG molecules, and the vast majority of these are of the subclass IgG1. There are currently 3 approved therapeutic IgG2 molecules; IgG2 molecules differ in the nature of disulfide bonding in the hinge region, yielding a mixed population of disulfide isoforms.[29] The hinge region of IgG2 molecules are of interest for HOS determination because the isoforms may differ in conformation leading to potential differences affecting the attributes of the molecule, such as product stability.

The conventional HDX-MS workflow has 4 major steps as shown in Figure 1. The labeling reaction is initiated by the dilution of protein sample into the deuterated buffer followed by the addition of pH 2.5 buffer after specific time points to quench the exchange reaction. To achieve localized HOS information, the deuterated sample is subjected to pepsin proteolytic digestion followed by liquid chromatography–mass spectrometry (LC-MS) analysis. In this work, the traditional HDX-MS pepsin digestion method was optimized in an effort to achieve increased sequence coverage and produce smaller overlapping peptides for more routine, better-quality HOS analysis of IgG molecules. Several enzymatic digestion strategies were investigated with commercially available proteolytic columns as shown in Figure 2. Ultimately, on our request, NovaBioAssays LLC co-immobilized 2 proteases, pepsin and type XIII protease from *Aspergillus*, in a single column for the first time, which was found to be the optimal configuration. An IgG2 molecule was used in the optimization process as a worst case scenario for biotherapeutic mAbs due to the disulfide complexity in the hinge region and general resistance to proteolysis. The method was then evaluated using IgG1 and IgG4 molecules to determine its general applicability to routine mAb characterization. The dual protease column was successfully used in the HDX-MS workflow to attain better sequence coverage with smaller, overlapping peptides with higher redundancy.

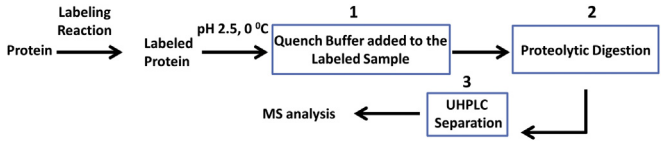

Figure 1. HDX-MS workflow highlighting the three regions for optimization: (1) Quench buffer conditions; (2) proteolytic digestion for better digestion efficiency; and (3) UHPLC gradient for better peptide separation and elution.

Experimental Section

Materials

Potassium phosphate di-basic (K_2HPO_4), potassium phosphate mono-basic (KH_2PO_4), formic acid, urea, and tris(2-carboxyethyl) phosphine HCl (TCEP.HCl) were purchased from Sigma (St. Louis, MO). Deuterium oxide (D_2O) was purchased from Cambridge Isotope Laboratories (Andover, MA). All IgGs were produced in Pfizer Inc. (Andover, MA or St. Louis, MO) using a CHO cell line with a defined media upstream culture process and downstream Protein A–based purification. Poroszyme Immobilized Pepsin Cartridge (2.1 × 30 mm) from ABI (Foster City, CA), Enzymate BEH Pepsin Column (2.1 × 30 mm, 5 μm) from Waters Corporation (Milford, MA), and type XIII column (2.1 mm × 30 mm) from NovaBioAssays LLC (Woburn, MA) were used.

Optimized HDX-MS Method

NovaBioAssays LLC kindly custom packed the prototype dual-protease column on request; here, 2 separately available proteases, pepsin and type XIII protease (from *Aspergillus*), were co-immobilized in a 1:1 ratio. Consequently, this dual protease column co-immobilized with pepsin and type XIII is now commercially available from NovaBioAssays LLC. A Waters HDX-MS Synapt G2-Si system with the LEAP (Carrboro, NC) automation manager was used for automated labeling, quenching, online proteolytic digestion, and LC/MS analysis.

The mAb sample was buffer exchanged into PBS buffer (10 mM, pH 7) using GE Vivaspin 500 centrifugal concentrators. The mAb (10 mg/mL) in PBS was diluted to 10 mg/mL in PBS buffer pH 7.5. The labeling reaction was initiated by diluting the 10 mg/mL mAb sample 1:25 in a deuterated buffer (10 mM PBS buffer, pH 7). The labeling reaction was quenched after respective time points (undeuterated, 0.5, 1, 10, 30, and 300 min) by adding 1:1 dilution of quenching buffer (1 M TCEP and 8 M urea in 100 mM PBS, pH 2.5) followed by a 60-s wait time and adding 25 μL of diluent (0.1% formic acid in H_2O) before injection onto the Waters HDX system for online proteolytic digestion using 75 μL/min flow rate of digestion solvent (0.1% [vol/vol] formic acid and 8 mM TCEP in water). Each time point was acquired in triplicate for analytical precision. The peptides were trapped on a Waters BEH 1.7 μm C18 trap and washed for 5 min to remove salts. Formic acid (0.1% [vol/vol]) in LC-MS grade water was used as mobile phase A and 0.1% (vol/vol) formic acid in LC-MS grade acetonitrile was used as mobile phase B. Peptides were separated on a Waters BEH C18