REVIEW

Hydrogen–Deuterium Exchange Mass Spectrometry as an Emerging Analytical Tool for Stabilization and Formulation Development of Therapeutic Monoclonal Antibodies

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Received 27 August 2014; revised 24 September 2014; accepted 26 September 2014

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.24224

ABSTRACT: The dynamic nature of the structure of monoclonal antibodies (mAbs) can be probed at a resolution of 5–20 residues using hydrogen–deuterium exchange mass spectrometry (H/D-MS). Recent studies using H/D-MS have shown that distinct regions of IgG1 mAbs experience significant changes in backbone dynamics in response to specific physicochemical alterations, varying solution conditions, or exposure to different environmental stresses. Tracking such changes in local dynamics may therefore serve as a key analytical tool, not only to monitor stability changes, but also to design improved, and more stable formulations of therapeutic mAbs in pharmaceutical dosage forms. This review article describes the H/D-MS method as applied to the analysis of formulations containing mAbs and summarizes recent studies monitoring changes in mAb local dynamics in response to chemical modifications, physical degradation, and presence of stabilizing and destabilizing excipients. Furthermore, the nature of the local dynamics of a highly conserved peptide segment in the C_H2 domain of IgG1 mAbs is reviewed, and the results are correlated with decreased pharmaceutical stability, supporting the identification of a common aggregation hotspot sequence in the F_c region of human IgG1 mAbs. In addition, unresolved challenges (and opportunities) in applying H/D-MS technology for stabilization and formulation development of mAbs are discussed. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci

Keywords: mass spectrometry; IgG antibody; monoclonal antibody; protein; formulation; protein aggregation; physical stability; physical characterization; molecular dynamics; chemical stability; excipients

INTRODUCTION

The IgG class of mAbs represents the largest category of therapeutic protein candidates currently under clinical development.[1,2] Antibodies are dynamic molecules with internal motions that are important for their biological function and stability. mAbs are large, multidomain, and multifunctional proteins that can be engineered to bind to a variety of therapeutic targets. An IgG molecule consists of two antigen binding (F_{ab}) regions, each containing a constant and variable domain from both the light and heavy chain, in the form of two arms attached by a proline-rich hinge region to the constant (F_c) region. The F_c region contains two constant Ig domains from each of two heavy chains including an N-linked glycosylation site. The F_c region is responsible for nonantigen binding biological activity and maintaining *in vivo* half-life. These regions are potentially flexible structures that lead to a wide range of possible conformations for IgG molecules. Dynamic motions of antibodies include whole molecule tumbling, rocking, and breathing motions along with bending, flexing, and rotational motions of the F_{ab} arms around the hinge region.[3–6] However, the interrelationships between regional and local dynamic motions and protein conformational stability are complex and is an active area of research.[7]

Therapeutic proteins such as mAbs are exposed to various environmental stresses during manufacturing, storage, and administration that can cause physical and chemical degradation. Hence, robust formulation strategies are needed to impart maximal stability to these proteins and to minimize degradation during long-term storage (e.g., across the shelf life). From a pharmaceutical development perspective, it is also a major challenge to predict the effects of product or process changes on the higher order structure and long-term stability of mAb drug products as part of comparability assessments.[8] One recent trend in the formulation development field is to explore the interrelationships between antibody dynamics, conformational stability, and pharmaceutical stability.[7,9–13] If direct relationships can be established between these variables, these relationships can lead to a better understanding of the implications of process and product changes on protein stability. This, in turn, would inform the design of improved mAb proteins and their formulations.

The global dynamics of mAbs have been studied by various techniques such as high-resolution ultrasonic spectroscopy, pressure perturbation calorimetry, red edge excitation shifts, and time-correlated single photon counting.[7,9,14] Data visualization tools such as empirical phase diagrams have been used to compare changes to the dynamic states of antibodies as a function of solution pH and temperature.[10] In addition, commonly available biophysical techniques such as circular dichroism, fluorescence spectroscopy, ultraviolet absorption spectroscopy,

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Journal of Pharmaceutical Sciences
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FTIR spectroscopy, and light scattering have been widely used to characterize the higher order structural integrity and conformational stability of proteins.[15] These biophysical methods provide global information about the overall dynamics and conformational stability of proteins.

One disadvantage of measuring global dynamics of antibodies is that there can be small scale, local changes in dynamics due to a variety of factors that may go undetected when averaged across the entire molecule.[12] However, one or a few such local changes in dynamics may be important for the stability or biological functions of an antibody. Hence, higher resolution analytical techniques capable of detecting and localizing small-scale changes in dynamics of an antibody are important for a more comprehensive assessment of changes in protein dynamics and stability. Two well-established high-resolution techniques that have previously been used to investigate local structural changes in smaller therapeutic proteins are nuclear magnetic resonance (NMR) spectroscopy[16,17] and X-ray crystallography.[18,19] Although great advances have been made in terms of applicability of these techniques to pharmaceutical development, practical limitations remain for the analysis of structural dynamics of an entire mAb molecule under formulation conditions. For example, some of the challenges in NMR analysis of mAbs are the requirement for isotopic labeling (^{15}N, ^{13}C, or ^{19}F) of the mAb and the difficulty of spectral crowding due to the large size of mAbs. X-ray crystallography data may not necessarily reflect antibody dynamics in complex solution buffers and the technique requires the generation of antibody crystals, which can sometimes be difficult to produce. Temperature factors for individual residues can be obtained as a measure of flexibility but are again limited to the solid state. Thus, there is a need for faster and more practical, higher resolution analytical techniques that are sensitive enough to detect changes in local structural dynamics of antibody molecules formulated in pharmaceutical dosage forms containing a wide range of protein concentrations and stabilizing excipients.

Hydrogen–deuterium exchange coupled to mass spectrometry (H/D-MS) potentially addresses this need.[20] This method utilizes the variable exchange rates of amide hydrogens along the protein backbone to provide information about the local dynamics of a protein. Hydrogen/deuterium exchange in proteins was first studied using gravimetric techniques by Hvidt and Linderstrom-Lang in the 1950s.[21] Since then, molecular sieve chromatography in tritiated water as well as several spectroscopic techniques such as infrared spectroscopy,[22–24] NMR,[25,26] and most recently mass spectrometry[27,28] have been used to measure hydrogen exchange kinetics in proteins. H/D-MS has shown promising results for analysis of dynamics of large proteins such as IgG antibodies with high sensitivity, low sample requirements and the ability to analyze proteins in complex formulation buffers.[12,13,20,29] Protease digestion using an acid resistant protease such as pepsin along with reduction of disulfide bonds at low pH has enabled peptide level analysis of mAb structure at a resolution of 5–20 amino acids.[28]

There has been a steady growth in the last five years in the number of reports describing the use of H/D-MS to explore dynamics of mAbs and antibody fragments. For example, H/D-MS has recently been used to assess changes in higher order structure of mAbs as a result of deglycosylation,[20] freeze–thaw and thermal processing,[30] chemical modifications,[31,32] posttranslational modifications,[29] formulating with pharmaceutical excipients and salts from the Hofmeister series,[12,13] targeted mutations,[33,34] and drug conjugation to free interchain cysteine residues.[35] This review paper summarizes the use of H/D-MS technique in advancing our understanding of the role of local dynamics in modulating antibody physical stability and implications for stabilization and formulation development of therapeutic mAbs.

FUNDAMENTALS OF AMIDE HYDROGEN EXCHANGE IN PROTEINS

When a protein is incubated in D_2O, its labile hydrogens will exchange with the solvent deuterium. There is nearly instantaneous exchange with deuterium by amine, carboxyl, and hydroxyl groups of amino acid residues, but these sites readily revert to hydrogen upon exposure to H_2O during LC separation (see below). In contrast, the amide hydrogens of the polypeptide backbone exchange much more slowly at rates strongly influenced by the folded structure of the protein.[21,36–39] Amide hydrogens within the folded β-sandwich structure of Ig domains that are solvent shielded and strongly hydrogen bonded to neighboring hydrogen bond acceptors are expected to exchange slowly. In contrast, the amide hydrogens on the surface or in the unstructured loop regions of an antibody that are solvent exposed and weakly hydrogen bonded are expected to exchange faster. The strength of hydrogen bonding of individual amide hydrogens can be assessed from the kinetics of hydrogen exchange at that location. The fastest exchanging amide hydrogens in a mAb can exchange in milliseconds at physiological pH[40,41] whereas the slowest exchanging amide hydrogen may take many months to exchange because the central domain regions of mAbs are known to have a tightly folded and stable structure. Hence, the difference in observed rates of deuterium exchange can provide valuable information about the local structure and dynamics of proteins such as mAbs.

Amide H/D exchange in the absence of any higher order structure is referred to as intrinsic or chemical exchange (i.e., k_{ch} in Eqs 1, 3 and 5). The rate of intrinsic exchange[42–44] can be expressed as

$$k_{ch} = k_A[H_3O^+] + k_B[OH^-] + k_W[H_2O] \quad (1)$$

where k_A, k_B, and k_W are the rate constants for acid-, base-, and water-catalyzed amide deuterium exchange reactions. The rate of intrinsic exchange is strongly influenced by solution pH, temperature, neighboring side chains, and solution components such as salts.[40,41] The intrinsic exchange reaction is predominantly base-catalyzed above pH 3.0 and predominantly acid-catalyzed below pH 2.3. Moving away from the minimum at a pH of approximately 2.5, the rate increases 10-fold for every unit of pH change. A temperature increase of $\sim 22^\circ$C has a similar effect.[45]

Proteins are known to exist as an ensemble of interconverting conformations in solution,[46–48] and each amide hydrogen may therefore exist in folded and unfolded conformations at any point in time. Amide hydrogen exchange by folded proteins can be described by one of two distinct models: the two-process model and the local unfolding model. The two-process model,[39] proposes that amides in both folded and unfolded states can exchange. Amides in the folded state exchange due to very small-scale fluctuations in the protein's native state. These fluctuations, though small, are sufficient to bring solvent and catalyst

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