Journal of Pharmaceutical Sciences xxx (2018) 1-10

Contents lists available at ScienceDirect

Journal of Pharmaceutical Sciences

journal homepage: www.jpharmsci.org

Pharmaceutical Biotechnology

Impact of Glycosylation on the Local Backbone Flexibility of Well-Defined IgG1-Fc Glycoforms Using Hydrogen Exchange-Mass Spectrometry

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ARTICLE INFO

Article history:
Received 24 December 2017
Revised 31 March 2018
Accepted 27 April 2018

Keywords:
stability
antibody
hydrogen exchange
mass spectrometry
glycosylation

ABSTRACT

We have used hydrogen exchange–mass spectrometry to characterize local backbone flexibility of 4 well-defined IgG1-Fc glycoforms expressed and purified from *Pichia pastoris*, 2 of which were prepared using subsequent *in vitro* enzymatic treatments. Progressively decreasing the size of the N-linked N297 oligosaccharide from high mannose (Man8-Man12), to Man5, to GlcNAc, to nonglycosylated N297Q resulted in progressive increases in backbone flexibility. Comparison of these results with recently published physicochemical stability and Fcγ receptor binding data with the same set of glycoproteins provide improved insights into correlations between glycan structure and these pharmaceutical properties. Flexibility significantly increased upon glycan truncation in 2 potential aggregation-prone regions. In addition, a correlation was established between increased local backbone flexibility and increased deamidation at asparagine 315. Interestingly, the opposite trend was observed for oxidation of tryptophan 277 where faster oxidation correlated with decreased local backbone flexibility. Finally, a trend of increasing C'E glycopeptide loop flexibility with decreasing glycan size was observed that correlates with their FcγRIIIa receptor binding properties. These well-defined IgG1-Fc glycoforms serve as a useful model system to identify physicochemical stability and local backbone flexibility data sets potentially discriminating between various IgG glycoforms for potential applicability to future comparability or biosimilarity assessments.

Introduction

Glycosylation is a widespread and complex post-translational modification, which introduces structural heterogeneity in recombinant therapeutic proteins.[1,2] Development and regulatory approvals of glycosylated mAbs, Fc-fusion proteins, antibody–drug conjugates, and antibody fragments have increased dramatically over the past 3 decades.[3,4] The IgG1 subclass of antibodies, the most abundant in human serum and the most common subclass for approved mAb therapeutics, has a conserved N-linked glycosylation site at N297 in the constant heavy chain (HC) of the Fc region.[5-9] N-glycan structures are known to modulate different effector functions of IgG antibodies including antibody-dependent cellular cytotoxicity (ADCC), complement dependent cytotoxicity (CDC), and antibody clearance by affecting FcγR, C1q, and FcRn binding, respectively.[10,11] Naturally occurring N-glycans of IgG1 antibody in the human serum are of the complex, biantennary type (consisting of 2 arms: one α-1, 3 arm, and one α-1, 6 arm) and contain a common 7 monosaccharide core (4 N-acetyl glucosamine (GlcNAc), and 3

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This article contains supplementary material available from the authors by request or via the Internet at https://doi.org/10.1016/j.xphs.2018.04.026.
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https://doi.org/10.1016/j.xphs.2018.04.026
0022-3549/

mannose (Man) residues).[12] Glycan heterogeneity in antibodies, arising from site occupancy (symmetric or asymmetric) and also from the type of glycans attached to the common core, governs the binding to various receptors thereby affecting the antibody's biological functions. For example, the presence of terminal and bisecting GlcNAc and the absence of fucose increases ADCC activity by increasing FcγRIIIa receptor affinity, whereas increasing terminal sialylation reduces ADCC activity.[13] Moreover, the presence of galactosylation promotes CDC by increasing interaction with C1q.[14] Hence, elucidation of mechanisms underlying these glycosylation effects is an active area of research that could lead to engineering antibodies having desired therapeutic effects.

The therapeutic efficacy of IgG mAb candidates depends on their structural integrity, conformational stability, flexibility, and biological functionality. N-glycosylation at N297 of the C_H2 domain influences the conformation, flexibility, aggregation propensity, and pharmacokinetic/pharmacodynamic properties of therapeutic mAbs, making it essential to evaluate the effects of glycosylation on product quality including physicochemical stability and biological efficacy.[13,15-18] Recombinant therapeutic mAbs requiring N-glycosylation are typically produced in mammalian expression hosts (e.g., CHO, SP20 and NSO cells).[19] Manufacturing conditions need to be tightly controlled to achieve a sufficiently high degree of reproducibility in terms of glycan heterogeneity during mAb production. However, glycan heterogeneity inevitably will still exist in mAbs produced in these recombinant expression systems that could potentially affect product quality.[20] For example, recombinant mAbs could have nonhuman glycans such as N-glycolylneuraminic acid instead of N-acetylneuraminic acid, or small amounts (ranging from 1% to 20%) of high mannose (HM, Man5-Man9) could affect their biological efficacy.[14,21,22] HM glycoforms of IgG have reduced serum half-life due to binding to mannose receptors, increased ADCC, and reduced CDC activities compared to antibodies containing complex fucosylated or hybrid glycans.[13,23] Hence, molecular heterogeneities like glycosylation are critical product quality attributes necessitating their close monitoring during therapeutic mAb production, upon manufacturing changes (comparability) and during biosimilar development. In addition, ensuring protein stability from manufacturing through patient administration is also a critical aspect of therapeutic protein drug development, where physicochemical degradation may lead to loss of potency, aggregation, and increased immunogenicity potential.[24,25] Hence, not only is it important to understand how glycosylation affects the pharmaceutical stability of antibodies but also to better understand the interrelationships between stability and the desired biological activity.

To this end, we generated a series of well-defined, nearly homogenous, IgG1-Fc glycoforms with serially truncated glycans. Previously, we analyzed these glycoproteins using multiple physical, chemical, and receptor binding assays as described in 4 manuscripts in the February 2016 issue of Journal of Pharmaceutical Sciences.[16-18,26] Results from this series of studies showed that the glycoform structure not only affected chemical degradation (especially deamidation of N315 and transformation of W277 into glycine hydroperoxide) but also led to different impurity profiles.[17] In addition, a correlation between physical stability and *in vitro* binding activity versus the size of the glycans was also observed. For example, HM-Fc and Man5-Fc had greater physical stability, higher apparent solubility, and stronger receptor binding than the GlcNAc and nonglycosylated N297Q-Fc.[16,18] These results suggested a strong correlation between decreased length of glycan and decreased apparent solubility, conformational stability, and *in vitro* receptor binding. Finally, these biochemical and biophysical data sets with the 4 well-defined IgG1-Fc glycoforms were further employed to develop an integrated mathematical model (using data mining and machine learning tools) for biosimilarity analysis.[26]

In this work, we measured the local backbone flexibility of these 4 IgG1-Fc glycoforms by hydrogen exchange–mass spectrometry (HX-MS). HX-MS provides information on higher-order structure and dynamics by monitoring the rate of backbone amide hydrogen exchange. Proteolysis following HX produces peptic peptides that are analyzed by liquid chromatography–mass spectrometry (LC-MS) to assess the deuteration level of each peptide (a measure of flexibility) as a function of time, so that localized differences are obtained at peptide resolution. HX-MS has been used extensively to investigate subtle higher-order structural changes and dynamics in mAbs as a consequence of aggregation, reversible self-association, oxidation, excipients, and point mutations.[27-30] However, only a limited number of studies have examined the effects of glycosylation on flexibility in IgG antibodies.[15,31-33] In this work, we correlate the effects of varying glycosylation on structural flexibility (especially in the C_H2 domain) as measured by HX-MS with our previously reported results on the overall conformational stability, chemical stability, and receptor binding profiles of 4 well-defined IgG1-Fc glycoforms as previously described. These correlations are discussed in the context of developing a better understanding of the interplay between glycosylation, stability, local flexibility, and biological function from a pharmaceutical perspective, especially applicability to future comparability or biosimilarity assessments.

Materials and Methods

Preparation and Initial Characterization of IgG1-Fc Glycoforms

We have reported the production of these Fc glycoforms in detail elsewhere.[18] Briefly, the IgG1-Fc glycoforms (HM-Fc, Man5-Fc, GlcNAc-Fc, and N297Q-Fc) were prepared by the expression of HM-Fc in a glycosylation-deficient strain of *Pichia pastoris* followed by *in vitro* enzymatic digestion of HM-Fc by a α-1, 2-mannosidase or endoglycosidase H to obtain the truncated Man5-Fc or GlcNAc-Fc. For the N297Q nonglycosylated IgG1-Fc, mutagenesis was used to remove the N-linked glycosylation. The proteins were pure (~99% by SDS-PAGE) and had minimal proteolysis products and high molecular weight species (1%-3%) after purification and enzyme truncation. The HM-Fc was heterogeneous, with N-glycans containing 8 to 12 mannose residues (Man8-Man12) at each N297 site; the major glycan was $Man_8GlcNAc_2$. The truncated Man5-Fc, GlcNAc-Fc glycoforms, and the nonglycosylated N297Q-Fc were well-defined and homogenous. With decreasing glycan size, the HM, Man5, GlcNAc glycoforms of IgG1-Fc and nonglycosylated N297Q-Fc mutant form a well-defined series of model glycoproteins. The purified glycoforms were stored at −80°C in 10 mM histidine buffer containing 10% sucrose at pH 6.0 until used for HX-MS studies.

Sample Preparation for HX-MS

For HX-MS studies, all 4 Fc glycoforms were thawed and then dialyzed into 20 mM citrate-phosphate buffer with ionic strength adjusted to 0.15 by NaCl at pH 6.0. Subsequently, the proteins were concentrated, as described elsewhere.[16] The final adjusted protein stock concentration of 1 mg/mL was determined by absorbance at 280 nm as measured by an UV–visible spectrophotometer (Agilent 8453, Palo Alto, CA). Components of all buffers including sodium phosphate and sodium chloride were purchased from Sigma-Aldrich (St. Louis, MO) while citric acid anhydrous and citric acid monohydrate were purchased from Fisher Scientific, all at the highest purity grade. Liquid chromatography grade acetic acid and phosphoric acid, tris (2-carboxyethyl) phosphine hydrochloride, porcine pepsin, guanidine hydrochloride, and deuterium oxide (99 + %D) were purchased from Fluka/Sigma-Aldrich.