Stability of Monoclonal Antibodies at High-Concentration: Head-to-Head Comparison of the IgG_1 and IgG_4 Subclass

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Received 24 June 2013; revised 7 September 2013; accepted 24 October 2013

Published online 26 November 2013 in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/jps.23788

ABSTRACT: Few studies have so far directly compared the impact of antibody subclass on protein stability. This case study investigates two mAbs (one IgG_1 and one IgG_4) with identical variable region. Investigations of mAbs that recognize similar epitopes are necessary to identify possible differences between the IgG subclasses. Both physical and chemical stability were evaluated by applying a range of methods to measure formation of protein aggregates [size-exclusion chromatography (SEC)–HPLC and UV340 nm], structural integrity (circular dichroism and FTIR), thermodynamic stability (differential scanning calorimetry), colloidal interactions (dynamic light scattering), and fragmentation and deamidation (SEC–HPLC and capillary isoelectric focusing). The impact of pH (4–9) and ionic strength (10 and 150 mM) was investigated using highly-concentrated (150 mg/mL) mAb formulations. Lower conformational stability was identified for the IgG_4 resulting in increased levels of soluble aggregates. The IgG_1 was chemically less stable as compared with the IgG_4, presumably because of the higher flexibility in the IgG_1 hinge region. The thermodynamic stability of individual mAb domains was also addressed in detail. The stability of our mAb molecules is clearly affected by the IgG framework, and this study suggests that subclass switching may alter aggregation propensity and aggregation pathway and thus potentially improve the overall formulation stability while retaining antigen specificity. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:115–127, 2014

Keywords: monoclonal antibody; protein formulation; protein aggregation; chemical stability; protein-protein interactions; high concentration; thermal analysis; physical stability

INTRODUCTION

Five classes of immunoglobulins (Ig) are known: IgA, IgD, IgE, IgG, and IgM, with IgG being the most abundant in circulation as well as the most used class as a therapeutic protein. IgGs are tetramers composed of two identical heavy chains (HCs) (50 kDa) and two identical light chains (LCs) (25 kDa) linked through interchain disulfide bonds. IgG is further divided into four subclasses (IgG_1, IgG_2, IgG_3, and IgG_4) based on small differences in the constant region of the HC. The major differences are found within the hinge region that connects two identical pairs of HC/LC. Both the length and the disulfide bonding pattern of the hinge vary among the subclasses. The flexibility of the antibody increases with the length of the hinge region in the order $IgG_3 > IgG_1 > IgG_4 > IgG_2$. The IgG_3 hinge is roughly four to five times as long as the other subclasses. The hinge of the IgG_1 antibody is 15 amino acids' long and contains two interchain disulfide bonds. IgG_2 and IgG_4 have the shortest hinge with only 12 amino acids. The numbers of interchain disulfides are four and two for the IgG_2 and IgG_4 subclass, respectively. Disulfides (intrachain) are also responsible for the compact domain-like structure of the molecule. When natively folded into the well-known "Y" shape, the constant domains CH1 and CL and the variable domains (VH and VL) comprise the antigen binding fragment (Fab). The complementarity determining region within the variable region determines the antigen specificity. The remaining domains of the HC (CH2 and CH3) form the Fc fragment (fragment crystallizable) of the molecule. The Fc fragment is responsible for effector function, which is the activation of the complement system and binding to Fc receptors. The efficiency of triggering effector functions varies with the diversity and flexibility of the hinge region, with IgG_3 being the most effective followed by IgG_1 and IgG_2. The IgG_4 antibody shows no capability of activating the complement cascade.[1–3] These differences are also observed with therapeutic mAbs, for example, Idusogie et al.[4] showed that complement recruitment could be weakened by substituting the IgG_1 framework in Rituximab® with an IgG_2.

The IgG_1 and IgG_2 antibody subclasses are the most abundantly used in the clinic, with 24 and 5 approved products, respectively. Currently, three IgG_4 products have been marketed, whereas the IgG_3 subclass has not yet been used clinically.[5] mAbs are used in the treatment of a wide range of diseases including cancer, inflammation, psoriasis, and bone diseases.[5–7] As with any other protein drug, the main obstacle in the development of mAbs is the handling of chemical and physical instabilities.[8] The most serious result of physical instability is protein aggregation. Protein aggregation cannot only affect the efficacy of the therapeutic treatment, but also induce serious adverse effects such as immunogenicity.[9–12]

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This article contains supplementary material available from the authors upon request or via the Internet at http://onlinelibrary.wiley.com/.

Journal of Pharmaceutical Sciences, Vol. 103, 115–127 (2014)

The desire for patient self-administration necessitates the use of either subcutaneous or intramuscular delivery routes. The volume restrictions (<1.5 mL) faced using these routes and the relative large amounts (several mg/kg) of mAb needed to achieve therapeutic effect requires development of highly concentrated (>100 mg/mL) liquid formulations.[13] Increasing protein concentration are generally believed to increase the risk of protein aggregation.[12,14] Solution factors such as solution pH and ionic strength can also affect the stability of a protein formulation. Both pH and ionic strength are recognized as some of the most critical factors determining the aggregation behavior of proteins.[11,12,14] Enhanced viscosity and solubility problems have also been described with high-concentration mAbs.[13,15–17] The physicochemical properties and aggregation behavior of a protein are not only dependent on solution conditions, but also on a protein's primary sequence and the folding into higher-order structures. Switching antibody subclass can thus potentially circumvent or improve these obstacles. Pepinsky et al.[17] showed that a simple replacement of the IgG_1 with an IgG_2 or IgG_4 framework in an anti-LINGO-1 Li33 mAb resulted in a remarkable increase in solubility. Two other studies reported increased aggregation propensity of the IgG_2 compared with the IgG_1 subclass, one under acidic conditions[18] and the other under physiological conditions.[9] The main driving force under acidic condition appears to be a lower stability of the CH2 domain in the IgG_2 subclass,[18] whereas an increased number of free thiol was reported as the main reason for the impaired stability of the IgG_2 subclass at physiological pH.[9] Arosio et al.[19] also investigated the stability of IgG_1 and IgG_2 mAbs under acidic conditions, but found no correlation between subclass and aggregation rate. A number of studies showed that the IgG_1 subclass is more resistant toward thermal denaturation compared with IgG_2 and IgG_4 molecules with the same variable region.[20,21]

The above-mentioned studies were all conducted under dilute solution conditions. The pronounced nonideality, decreased intermolecular distance, and protein–protein interaction patterns at high concentration can affect the stability at high concentration. A number of studies have shown distinct aggregation behavior of proteins at low versus high concentration.[22,23] It is thus important to address aggregation issues of different IgG subclasses at high protein concentration. To the best of our knowledge, no study exists as yet that has investigated the stability of mAbs of IgG_1 and IgG_4 with identical variable region. Investigating mAbs that recognize the same antigen is important to identify the stability behavior of the subclass itself. Two mAbs, one of the IgG_1 and one of the IgG_4 subclass, were used for this purpose. The sequence identity in the constant region between the mAbs is approximately 95%, with the main differences found in the hinge region. The aim of the present study was to investigate the impact of pH and ionic strength on conformational, colloidal, and chemical (fragmentation and deamidation) stability of the IgG_1 and IgG_4 subclass using a broad range of analytical techniques.

MATERIALS AND METHODS

Model Proteins

Two mAbs were used in this study, manufactured by Novo Nordisk A/S. The antibodies B72.3 IgG_1 and IgG_4 recognize the antigen tumor-associated glycoprotein (TAG-72) and have been developed as a murine antibody of the IgG_1 subclass. The variable domains were cloned and transferred to human IgG_1 and IgG_4 constant domains. The IgG_4 mAb contains a single amino acid substitution within the hinge region (S241P) compared with the wild-type IgG_4. This mutation serves to avoid formation of half-antibodies normally observed for the IgG_4 subclass.[24] The mAbs were purified using protein A affinity chromatography followed by concentration and buffer exchange (10 mM histidine buffer pH 6.5) using tangential flow filtration.

The isoelectric points (p*I*s) of the IgG_1 and IgG_4 mAb were 8.6 and 7.3, respectively [determined from the main species using capillary isoelectric focusing (cIEF)]. The effective p*I*, determined from the potential zeta potential as a function of pH, is approximately 1 pH unit lower (7.5 for the IgG_1 and 6.5 for the IgG_4 mAb).

Sample Preparation

Low ionic strength buffers were prepared by dissolving 1.2 mM of each buffer species (citric acid/monobasic phosphate) (Sigma–Aldrich, St. Louis, Missouri) in Milli-Q water. The target pH was reached by titrating with sodium hydroxide. The ionic strength was adjusted to 10 mM with sodium chloride (NaCl) (Merck, Darmstadt, Germany). Samples at the pH of interest (4.0–9.0 ± 0.1) were prepared using Millipore Amicon Ultracentrifugation tubes (Billerica, Massachusetts) with a molecular weight cutoff of 10 kDa. After each buffer exchange and concentration to greater than 150 mg/mL, the pH was measured to confirm a successful buffer exchange. At pH extremes (pH 4.0, 8.0, and 9.0), either lower (for pH 4.0) or higher (for pH 8.0 and 9.0), pH buffers was used to obtain the desired pH at 150 mg/mL protein concentration. This was necessary to counteract the significant buffer capacity of the protein itself as well as the impact from the Donnan effect, thereby changing the ion composition and solution pH.[25] Higher ionic strength samples (150 mM) were prepared by adding a small amount of 5 M NaCl.

The protein concentration of the samples was determined using UV spectrometry using an extinction coefficient of 1.43 mL mg^{-1} cm^{-1} at 280 nm for both mAbs.

Stability Study

Three individual samples at each pH and ionic strength were added to 1.5 mL type I glass cartridges, defined according to Ph.Eur. 3.2.1. The cartridge was closed in one end with a bromobutyl rubber plunger (type I rubber as defined in Ph.Eur. 3.2.9) and an aluminum cap with laminate bromobutyl rubber disc inserted in the other. The rubber plunger was raised to exclude any air inside the cartridge. The vials were closed to avoid any evaporation during incubation. The vials were placed in a heating cabinet at 40°C. Samples were withdrawn after 0, 2, 4, and 8 weeks of incubation. All samples were measured in triplicate unless stated otherwise. Results for the pH 4 samples are not shown because of the formation of a gel within 2 weeks of storage.

Evaluation of Physical Stability

Size-Exclusion Chromatography

Withdrawn samples were analyzed using a HPLC system with a TSKgel $G3000SW_{XL}$ (Tosoh Bioscience, Tokyo, Japan) gel filtration column installed. Each sample of 150 mg/mL was diluted (1:20) in a buffer containing 50 mM sodium phosphate,