Temperature-Ramped Studies on the Aggregation, Unfolding, and Interaction of a Therapeutic Monoclonal Antibody

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ABSTRACT: Investigations on thermal behavior are essential during the development of therapeutic proteins. Understanding the link between thermal unfolding and aggregation might help to minimize conformational and colloidal instabilities. In this study, a therapeutic monoclonal antibody and its Fab and Fc fragments were investigated. The apparent melting temperature of a protein and its onset were determined by differential scanning fluorimetry. Temperature-ramped turbidity measurements were performed to assess the temperature of aggregation, where large protein particles occurred. The formation of small aggregates was monitored and the interaction parameter k_D at low, ambient, and high temperature was calculated by temperature-ramped dynamic light scattering. Transformation of k_D into A_2^* based on literature findings allowed the interpretation of net repulsive or attractive conditions. Repulsive net charges at low pH increased the colloidal stability, although a reduction of the conformational stability was observed. At neutral conditions and in the presence of salt, unfolding was followed by precipitation of the protein. A sharp decrease of k_D and negative A_2^* values suggest that the aggregation was driven by hydrophobic interactions. Thus, the presented methods described and explained the thermal behavior of the protein and demonstrated their value for the development of pharmaceutical protein products. © 2013 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 103:445–455, 2014

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INTRODUCTION

The therapeutic and commercial success of protein drugs, especially monoclonal antibodies (MAbs), is well known today. The development of new drugs, biosimilars of marketed products, or other forms such as antibody drug conjugates, is still challenging because of the outstanding complexity of the stability and function of the protein molecule.[1] MAbs commonly face different types of instability reactions, including chemically altered material, changed conformational structures, or the formation of native or nonnative protein aggregates.[2–5] All of these instabilities can reduce the efficiency and safety of the product. For example, immunogenic reactions in patients are assumed to be caused by "altered protein," although clear evidence is still lacking, and the topic is in hot discussion.[6,7] It is reasonable to minimize the occurrence of unwanted altered protein during the development of a protein drug. Spectroscopic methods such as fluorescence, ultraviolet–visible (UV–Vis), circular dichroism, and Fourier-transform infrared spectroscopy are typically applied to characterize the conformational structure of the protein.[8] Additionally, a thermal analysis by differential scanning calorimetry (DSC) or differential scanning fluorimetry (DSF) reveals the apparent melting temperature (T_m) of a protein, which is commonly linked to the overall thermal stability of the protein.[9–12] Because of the vast distribution of particle size, many methods are necessary to describe protein aggregates comprehensively. Typically, high-performance size-exclusion chromatography (HP-SEC) and light scattering techniques are applied for small, soluble aggregates; nanoparticle tracking analysis, light obscuration, and microflow imaging are applied for subvisible particles; and visual inspection is applied for large particles, to name some prominent examples.[5,13]

A net attraction between protein molecules leads to aggregation, although multiple individual forces are involved. The interaction potential is composed of long (Coulomb) and short-range forces (van der Waals, solvation and hydrogen bonds, hard-sphere repulsion/excluded volume, and other specific interactions).[14,15] Instead of assessing each single force, a common approach is the determination of the osmotic second virial coefficient A_2 from the parametric expansion of the osmotic pressure (also referred to as B_{22}). A_2 describes the deviation from the ideal solution due to overall pairwise molecular interactions between the particles.[16] Although crystallization and solubility were thoroughly investigated in relation to A_2 for many proteins,[17–25] research on MAbs focused mainly on the effect of intermolecular interactions at the low concentration on the viscosity of highly concentrated MAb solutions. Saito et al.[26] demonstrated that A_2 is dependent on the surface properties of MAbs, and a high negative A_2 value correlated with a high viscosity of concentrated MAb solutions, and an increased tendency of the MAb for aggregation due to the attractive interactions. Also, a strong correlation between A_2 and the viscosity of highly concentrated MAb solutions was found by Connolly et al.[27] They concluded that weak interactions are responsible because the net charge of the MAb was in no correlation.[27] In their work, the assessment of A_2 via static light scattering (SLS) or analytical ultracentrifugation (AUC) is of low throughput and other methods such as self-interaction chromatography have been proposed to reduce time and material consumption.[24,28–31]

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Yadav et al.[32] recently published a different approach to determine A_2 based on measurements only using a dynamic light scattering (DLS) instrument. With careful consideration of the attenuation factor, the recorded scatter intensity signal is used to create Debye plots to calculate A_2. Although this elegant approach enables A_2 measurements in a single machine, it is limited by a low throughput and cannot be adopted to well plate readers. On the contrary, the diffusion interaction parameter k_D, which is easily accessible via DLS, is used as an alternative screening parameter. The k_D value can be expressed as [33]

$$k_D = 2A_2M - (\zeta_1 + 2\upsilon_{sp}) \quad (1)$$

where A_2 is the osmotic second virial coefficient, M is the molar mass of the protein, ζ_1 is the coefficient from the linear term of the virial expansion of the concentration-dependent friction coefficient, and υ_{sp} is the partial specific volume of the protein. Thus, the k_D value consist of a first, thermodynamic part expressed by A_2 and a second, hydrodynamic part. To determine k_D, the mutual diffusion coefficient D is determined for several dilutions of the protein sample. From the slope of the linear fit to the data points, k_D can be determined based on the following equation:

$$D = D_0(1 + k_D c) \quad (2)$$

where D is the mutual diffusion coefficient, D_0 is the diffusion coefficient at infinite dilution, and c is the protein concentration.

Yadav et al.[34] investigated the interactions of MAbs at low ionic strength and suggested that close to the isoelectric point (p*I*), specific attractive forces are responsible for self-association and also lead to high viscosity in high MAb concentrations, although a correlation to k_D was not found under these latter conditions. Chari et al.[35] involved k_D measurements to demonstrate that at high MAb concentrations and low ionic strength, both long- and short-range electrostatic interactions are responsible for viscosity effects. Although long-range electrostatic interactions were responsible for MAb aggregation at low concentration, Kumar et al.[36] found that at high MAb concentrations, short-range hydrophobic interactions predominate the aggregation kinetics. Although Arzenšek et al.[37] intensively investigated the effects of pH and ionic strength on k_D by the addition of sodium chloride, Rubin et al.[38] recently studied the aggregation of a MAb with respect to the ionic strength from salts of the Hofmeister series. They found that a lower k_D value is suggestive for a lower relative colloidal stability of the MAb.[38] However, because of the hydrodynamic component, the sign of k_D does not indicate net attractive or net repulsive intermolecular interaction.[26] Lehermayr et al.[39] and also Connolly et al.[27] present a linear fit of the data to transform k_D into A_2. In this article, a linear fit was performed on the united data set of A_2 and k_D values published in literature on MAbs to obtain an equation to transform the interaction parameters. This equation was used to estimate the thermodynamic interaction parameter (A_2^*) and thus the net attraction or net repulsion.

To understand the role of intermolecular interactions during the process of thermal unfolding and aggregation of MAbs, k_D and A_2^* values were investigated at low, ambient, and elevated temperature by DLS. The absolute values and their change upon heating were used to interpret the results from DSF regarding the conformational stability and the exposure of hydrophobic patches upon unfolding. The results from the DLS and temperature-ramped turbidity experiments were linked to characterize and interpret the aggregation behavior. Additionally, the Fab and Fc domains were isolated to study the influence of the individual domains on the overall stability of the MAb. A strong effect of pH and ionic strength was observed. At the low pH of 5.0 and low ionic strength, a high k_D value was observed, suggesting strong electrostatic repulsion, which prevented the protein from precipitation. Despite this colloidal stabilization, a reduced conformational stability was found with respect to the negative shift of the T_m value compared with the neutral pH. At pH 7.2 and 5.0 in the presence of salt, lower k_D values were found. Furthermore, different aggregation behaviors for Fab and Fc or the full MAb were observed. In the case of Fab, k_D values did not change with increasing temperatures until presumably minimal perturbation of the structure below the onset temperature of unfolding resulted in substantial aggregation. In the case of Fc and the full MAb, unfolding and aggregation coincided at similar temperatures and were associated with a reduction of k_D, suggesting that the conformational change induced new interaction spots. At the neutral pH or when the charges were shielded in the presence of a salt at the low pH, precipitation of the protein occurred.

MATERIALS AND METHODS

MAb Fragmentation and Protein Formulation

A therapeutic MAb of immunoglobulin G1 type produced in CHO cells was digested with 0.1% (w/w) of papain from papaya latex (Sigma–Aldrich, Steinheim, Germany) in a 40 mM histidine buffer containing 10 mM cysteine and 2 mM disodium ethylenediaminetetraacetic acid at pH 7.0. The mixture was incubated for 24 h at 37°C, and the reaction was stopped by the addition of an aqueous iodoacetamide solution to reach a concentration of 40 mM iodoacetamide. To isolate the fragments, the mixture was purified by chromatography using an ÄKTA purifier 10 system (GE Healthcare, Uppsala, Sweden) and ultrafiltration steps. First, all Fc-carrying species were retained by protein A affinity chromatography using a 5 mL Pierce protein A cartridge (Thermo Fisher Scientific, Bonn, Germany). Second, SEC using a Sephacryl 16/60 column (GE Healthcare) was performed to separate Fc from residual full MAb or partly fragmented MAb. Finally, the protein was dialyzed by means of Vivaspin 20 tubes with a 10 kDa molecular weight cutoff polyethersulfone membrane (Sartorius Stedim Biotech, Göttingen, Germany) to remove all low-molecular-weight species with the target formulation buffer (10 mM phosphate at pH 5.0 and 7.2, with or without 140 mM sodium chloride and 280 mM mannitol). The pH of the samples was adjusted and filtration was performed using a 0.2 μm syringe filter (Pall, Port Washington, New York). The protein concentration was determined using an extinction coefficient of 1.49 mL g^{-1} cm^{-1}. Isoelectric focusing using a Servalyt Precotes gel (Serva Electrophoresis, Heidelberg, Germany) resulted in p*I*s between 8.3 and 9.5 for the full MAb, 9.5 and 10.7 for Fab, and 6.9 and 7.8 for Fc. The purity of the isolated Fab and Fc fragments was confirmed by hydrophobic interaction chromatography using a TSKgel Phenyl-5PW column (Tosoh Bioscience, Stuttgart, Germany) on an Agilent 1200 series high-performance liquid