Mechanism of Reversible Self-Association of a Monoclonal Antibody: Role of Electrostatic and Hydrophobic Interactions

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ABSTRACT: Reversible self-association of protein therapeutics, the phenomenon of formation of native reversible oligomeric species as a result of noncovalent intermolecular interactions, can add additional manufacturing, stability, delivery, and safety complexities in biopharmaceutical development. Its early detection, characterization, and mitigation can therefore contribute to the success of drug development. A variety of structural and environmental factors can contribute to the modulation of self-association with mechanisms still elusive in some cases due to the inherent structural complexity of proteins. By combining the capabilities of dynamic and static light scattering techniques, the modulatory effects of a variety of solution conditions on a model IgG1's (mAbA) intermolecular interactions have been utilized to derive mechanism of its self-association at relatively low-protein concentration. The analysis of the effect of pH, buffer type, Hofmeister salts, and aromatic amino acids utilizing light scattering supported a combined role of hydrophobic and electrostatic interactions in mAbA self-association. Fitting of the data into the equilibrium models obtained from the multiangle static light scattering provided the enthalpic and entropic contributions of self-association, highlighting the more dominant effect of electrostatic interactions. In addition, studies of the Fab and Fc fragments of mAbA suggested the key role of the former in observed self-association. © 2014 Wiley Periodicals, Inc. and the American Pharmacists Association J Pharm Sci 104:577–586, 2015

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INTRODUCTION

During various stages of manufacturing, fill-finish, storage, transportation, and delivery, protein therapeutics often get exposed to a number of environmental stresses including extreme pH, freeze–thaw, shear, agitation, temperature excursions, and interactions with a variety of container-closure surfaces.[1,2] Such stresses, in combination with the high-structural complexity and inherent marginal stability of protein therapeutics, can potentially lead to a variety of physical and chemical degradations. Although the former mainly occur due to conformational (e.g., unfolding), colloidal (e.g., protein–protein interactions), or interfacial (e.g., adsorption to interfaces) instabilities, the latter refers to modifications of the covalent bonds such as oxidation, deamidation, isomerization, and disulfide bond shuffling.[3,4]

Among the variety of potential degradation pathways, aggregation is perhaps the most common and well-recognized, spanning a wide range of molecular sizes, morphologies, and compositions.[5] Conventionally, aggregation refers to the formation of nonnative oligomeric species as a result of interactions among "altered" monomeric units, induced via either conformational or chemical modifications.[6] Such aggregates have received wider attention and have been extensively studied and characterized due to their potential deleterious effects such as loss of efficacy and induced immunogenicity of protein therapeutics.[7,8] Compared with nonnative aggregation, less attention has been paid to the formation of native and reversible aggregates as a result of noncovalent interactions among "native" monomeric units, a phenomenon often referred to as "reversible self-association."

Although nonnative aggregates can pose immunogenicity risks, native reversible-associated species are more of a concern from the manufacturing and delivery perspectives. Such species can, for example, lead to increased viscosity resulting in clogging of the lines and filters[9,10] or impact injectability with potential pain upon injection when administered to patients.[11,12] In more rare cases, reversible-associated species can impact bioactivity and pharmacokinetic properties[13,14] or depending on the structural and environmental conditions, transform to irreversible aggregates via further covalent linkages.[15]

A variety of molecular and structural properties including net charge, charge distribution, charge heterogeneity, and surface hydrophobicity can potentially contribute to protein–protein interactions and reversible self-association thereof.[16] The specificity of such structural effects has been highlighted through mutation studies.[16,17] Solution conditions such as pH, ionic strength, temperature, and excipients are known to modulate and shift the equilibrium between monomeric and oligomeric species.[10,11,18–22] Understanding the mechanism of reversible self-association through traditional structural

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analyses is often difficult due to complexity and large size of protein molecules. Alternatively, a thermodynamic understanding through modulatory effects of solution properties on the type and extent of protein–protein interactions can provide a basis for the mechanisms of self-association.

We previously demonstrated the application of a multi-technique approach with dynamic and composition gradient static light scattering along with analytical ultracentrifugation for the detection and assessment of reversible self-association utilizing a model monoclonal antibody.[23] Herein, the underlying mechanism of self-association observed at relatively low-protein concentration for the aforementioned monoclonal antibody (referred to as mAbA) has been investigated utilizing the dynamic and static light scattering techniques. The protein's response to key solution parameters such as pH, salt type and concentration, and temperature has been examined and the corresponding energetics of interactions (i.e., enthalpic and entropic contributions) has been calculated. A mechanistic pathway for mAbA self-association is then depicted based on the collective observations of the impact of solution parameters. Finally, studies on Fab and Fc fragments of mAbA and their association propensities have been performed to identify the specific structural regions responsible for the observed self-association.

MATERIALS

mAbA Sample Preparation and Concentration Determination

The mAbA samples for all experiments were prepared by dialyzing the stock solution (15 mg mL^{-1}) against either 10 mM histidine/histidine–HCl buffer or 10 mM citrate-phosphate buffer exhaustively over 24 h at refrigerated temperatures. Thermo scientific (Waltham, Massachusetts) Slide-A-Lyzer dialysis cassettes with molecular weight cutoff of 10 kDa were used for dialysis. The concentration of mAbA after dialysis was determined by UV absorption spectroscopy ($A_{280\ nm}$) using an experimentally determined $E_{1\ cm}$ 0.1% of 1.54 mL mg^{-1} cm^{-1}. All experiments were performed over a concentration range of 1 or 2 to 10 mg mL^{-1}, unless stated otherwise. Salt stock solutions of 500 mM were prepared in 10 mM histidine buffer, pH 6, and were spiked into mAbA solution (already in 10 mM histidine buffer) at appropriate levels to generate salt-containing solutions with 10–150 mM salt concentrations. All samples were filtered using a 0.22-μm Amicon filter (Millipore, Billerica, Massachusetts) before use in both dynamic and static light scattering experiments.

Generation of Fab and Fc Fragments by Papain Digestion of mAbA

mAbA was dialyzed into 20 mM sodium phosphate, 10 mM Ethylenediaminetetraacetic acid (EDTA), pH 7.0 buffer. A digestion ratio of approximately 8 mg of IgG to 0.25 mL of settled resin was used. Immobilized papain (Thermo Scientific) was washed with a 20× volume of 20 mM sodium phosphate, 10 mM EDTA, 20 mM cysteine–HCl, pH 7.0. The papain resin was vacuum filtered until semi-dried. Prior to digestion, cysteine–HCl (Thermo Scientific) was added to the mAbA solution to a final concentration of 20 mM. The washed semi-dry resin was added to mAbA solution and mixed end-over-end at 37°C for 24 h. The Fab and Fc solutions were recovered by vacuum filtration. The resin was washed with two volumes of 20 mM sodium phosphate, pH 7.2, and was combined with the filtrate. The final Fab and Fc solutions were then dialyzed into 1× phosphate-buffered saline (PBS; Life Technologies, Carlsbad, California), pH 7.2, prior to a final dialysis in 10 mM histidine buffer, pH 6, for dynamic light scattering (DLS) studies.

Purification of Fab and Fc Fragments by MabSelect SuRe Chromatography

Separation of the Fab and Fc fragments was performed on a 5-mL MabSelect SuRe HiTrap column with a binding of 20 mg mL^{-1} resin and a flow rate of 5 mL min^{-1}. The Fc domain binds to MabSelect SuRe and elutes at low pH whereas the Fab does not. The Fc fraction was pH neutralized and subsequently dialyzed into 1× PBS (Life Technologies), pH 7.2. The Fab domain was further purified by gel filtration over a Superdex 200 column. Both Fab and Fc final solutions were evaluated for purity by HPSEC in which 97.2% and 99.3% purity levels were measured, respectively (data not shown).

METHODS

Dynamic Light Scattering

The hydrodynamic diameter of mAbA particles and corresponding Fab and Fc fragments were analyzed using a high-throughput 384-well plate DynaPro DLS instrument (Wyatt Technology, Santa Barbara, California) equipped with a 633-nm laser. The scattered light was monitored at 173° to the incident beam and autocorrelation functions were generated using a digital autocorrelator. A 30-μL volume of sample was loaded into each well and the plate was spun at 2000 rpm for 2 min to remove any air bubbles. Samples were run in triplicate and data were collected using 10 5-s acquisitions per sample. The hydrodynamic diameter was calculated from the diffusion coefficient based on the Stokes–Einstein equation using the method of cumulants.[24] Temperature was controlled using a Peltier-based controller and the hydrodynamic diameter data were collected upon adequate equilibration at the corresponding 5°C, 25°C, and 37°C. Finally, viscosity corrections were applied where appropriate (data not shown).

Modeling of the DLS data was performed employing weighted average apparent hydrodynamic diameters (D_h) of 11.7 and 45.7 nm for monomeric ($D_{h,mon}$) and hexameric ($D_{h,hex}$) species, respectively. The monomeric size is the true hydrodynamic size of mAbA[23] from which the hexameric size was calculated based on Eq. (1):

$$D_{h,hex} = \frac{(6D_{h,mon}) + (6^{1/3}D_{h,mon})}{2} \quad (1)$$

The estimated hexameric size above represents an average of the maximum and minimum hydrodynamic sizes a hexameric species can possess. The hydrodynamic sizes of monomers and hexamers, multiplied by their respective fractional presence, at known total concentration were then used to estimate the monomeric and hexameric concentrations. The concentration of monomer can be calculated according to Eq. (2):

$$[Mon] = [Total][1 - (D_{h,obsevered} - D_{h,mon})/(D_{h,hex} - D_{h,mon})] \quad (2)$$

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