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Trimerization Dictates Solution Opalescence of a Monoclonal Antibody

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ABSTRACT

Opalescence, sometimes observed in antibody solutions, is thought to be mediated by light scattering of soluble oligomers or insoluble particulates. However, mechanistic features, such as stoichiometry and self-association affinity of oligomeric species related to opalescence, are poorly understood. Here, opalescence behavior of a monoclonal antibody (mAb-1) solution was studied over a wide range of solution conditions including different protein concentrations, pH, and in the presence or absence of salt. Hydrodynamic and thermodynamic properties of mAb-1 solutions were studied by analytical ultracentrifugation and dynamic light scattering. Opalescence in mAb-1 solutions is pH and concentration dependent. The degree of opalescence correlates with reversible monomer−trimer equilibrium detected by analytical ultracentrifugation. Increased trimer formation corresponds to increased opalescence in mAb-1 solutions at higher pH and protein concentrations. Addition of NaCl shifts this equilibrium toward monomer and reduces solution opalescence. This study demonstrates that opalescence in mAb-1 solutions does not arise from the light scattering of monomer or random molecular self-associations but is strongly correlated with a specific self-association stoichiometry and affinity. Importantly, at pH 5.5 (far below isoelectric point of mAb-1), the solution is not opalescent and with nonideal behavior. This study also dissects several parameters to describe the hydrodynamic and thermodynamic nonideality.

Introduction

Antibody drugs, as a major class of protein therapeutics, play a crucial role in providing cures for cancers, infectious diseases, and autoimmune diseases. Since the approval of the first monoclonal antibody (mAb) product, OKT-3, in 1986, >40 antibody drug products (or product variations) have been commercialized.[1] The significant increase in the number of commercial antibody products and antibody therapeutic candidates in commercial development is largely due to their high target specificity (leading to minimal side effects). The significant progress in the development of antibody therapies has been facilitated by a better understanding of the antibody molecular properties and solution behaviors, which are generally more predictable than other types of protein products.[2] Nonetheless, the solution behavior of antibodies can vary significantly, although they are structurally similar.

Antibody aggregation is one of the critical challenges encountered during drug product development. Presence of even small amounts of protein aggregates in a drug product may potentially affect its biologic activity, safety, immunogenicity, and aesthetics.[3,4] Protein aggregates can form through different pathways and take a variety of forms.[5] These can be broadly divided into soluble aggregates quantifiable by the traditional size exclusion chromatography, and insoluble particulates, which can be visible or subvisible, depending on their sizes.[6] Insoluble protein aggregates may lead to opalescence, turbidity, and gelation.[7]

Protein opalescence has been observed and reported in many antibody studies. Specific examples include human IgG,[8,9] IgG4,[10] IgG2,[11] and many IgG1 monoclonal antibodies.[12-16] In addition to the potential aggregation-related issues, opalescent protein solutions can further lead to phase separation[11,14] and interference with routine characterization of protein solution properties,[10] presenting challenges for protein drug development.

A limited number of studies have been conducted on the causes of opalescence in protein solutions. One prevailing explanation is the concentration-dependent protein self-association and the consequent increase in light scattering.[12-14] Protein self-association is generally a result of weak protein−protein interactions. Therefore,

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factors such as pH, excipients, and ionic strength can easily affect protein–protein interactions and change the degree of opalescence as demonstrated in this study. However, the exact linkage of opalescence with the degree and reversibility of protein self-association remains to be better understood.

In this study, we report observation of a pH- and concentration-dependent opalescence of an IgG2 monoclonal antibody (mAb-1) solution. Three fundamentally different biophysical approaches were used to characterize the protein solution properties—sedimentation velocity—analytical ultracentrifugation (SV-AUC), dynamic light scattering (DLS), and sedimentation equilibrium—analytical ultracentrifugation (SE-AUC). Over a wide range of protein concentrations, different pH, and presence or absence of NaCl, we found that (i) at a pH (5.5) far below the isoelectric point (pI = 8.3), mAb-1 shows a concentration-dependent nonideal behavior; (ii) mAb-1 shows greater opalescence at a pH (8.5) close to its pI and in the absence of NaCl; (iii) this opalescence is strongly correlated with a concentration-dependent formation of a reversible trimer; and (iv) presence of NaCl shifts the monomer–trimer equilibrium toward monomer, dramatically reducing the degree of opalescence; (ii) to (iv) suggest that mAb-1 trimerization dictates its solution opalescence and provide insight into opalescence reduction.

Materials and Methods

A monoclonal antibody of IgG2 subclass, also referred as mAb-1, was manufactured at Pfizer St Louis laboratories and used throughout the studies. Concentrated stock protein solution was stored frozen in a 20 mM acetate buffer before use. mAb-1 has a molecular weight of 147.3 kDa with a theoretical pI of 8.3. All other chemicals used are of analytical grade.

Buffer and Sample Preparation

Different buffering agents: acetate, phosphate, and Tris, were prepared at 20 mM for pH 5.5, 7.2, and 8.5 (measured at 5°C) buffer solutions, respectively. All protein samples were prepared by dialysis with proper buffer solutions and adjusted to different protein concentrations with the dialysate. For higher concentration experiments, the protein samples were concentrated by Millipore Centricon with a 50-kDa cutoff filter. Protein concentrations were determined by UV absorption at 280 nm using an extinction coefficient of 1.47 $(mg/mL)^{-1}$ $(cm)^{-1}$.

Turbidity Measurement

Turbidity of mAb-1 solution was measured using a HACH 2100AN Turbidimeter (Loveland, CO). All samples were filtered with a 0.22-μm Millex-GP syringe filter unit immediately before the measurement. The instrument was calibrated using nephelometric turbidity unit (NTU) standards (20, 200, 1000, and 4000 NTU) and a proper control (40 NTU). The light scattering data were collected at designated temperatures and at 90° on the turbidimeter and then converted to NTU.

Particle Measurement

The subvisible particle counts were determined using an HIAC 9703 Liquid Particle Counter (Beckman Coulter, Indianapolis, IN) equipped with an HRLD-150 sensor and 1-mL syringe. The system was flushed with particle-free Milli-Q water and verified with 15-μm Duke latex standard before the analysis. For each sample, 4 consecutive measurements were performed where the first measurement was discarded. The data for the other three measurements were collected using PharmSpec v3.0, and the cumulative concentration (counts/mL) for relevant particle sizes (10 and 25 μm) was recorded.

Sedimentation Velocity

SV experiments were performed using a Beckman XLA analytical ultracentrifuge. Lower protein concentration samples (0.1-5.0 mg/mL) were loaded into Epon charcoal-filled 2-sector centerpieces with a 12-mm path length. Higher concentration samples (5.0-10.0 mg/mL) were loaded into 3-mm path length cells. For cells with 12-mm centerpiece, 450 μL of reference buffer was loaded into the reference sectors and 440 μL of sample was loaded into the sample sector. For cells with 3-mm centerpiece, 110 μL of reference buffer was loaded into the reference sector and 107 μL of sample was loaded into the sample sector. The experiments were performed at 5°C and 40,000 rpm. The samples and instrument were equilibrated at 5°C for at least 2 h before initiation of the experiment. The data were collected at 280 nm for samples with concentration between 0.1 and 1 mg/mL, 295 nm for sample with concentration between 2 and 5 mg/mL, and 300 nm for samples with 7-10 mg/mL. The raw SV data were analyzed using both DCDT(+)[17] and SEDFIT,[18] and the sedimentation coefficients were corrected to standard conditions (20°C, in H_2O).

Dynamic Light Scattering

The hydrodynamic radius of mAb-1 was analyzed using a Malvern Zetasizer Nano Series (Malvern, UK) equipped with a 633-nm laser. The precooled samples and instruments were equilibrated at 5°C for 5 min before the data collection. The scattered light was monitored at 173° to the incident beam, and autocorrelation functions were generated using a digital autocorrelator. The data were collected in triplicates and analyzed using Malvern DTS software package, where the buffer viscosity was entered based on independent viscosity measurements by *m-VROC* Viscometer (RheoSense, San Ramon, CA).

Sedimentation Equilibrium

Samples were prepared and loaded into Epon charcoal-filled 2-sector centerpieces as described previously. For lower concentration samples (0.1-10 mg/mL), 150 μL of reference buffer was loaded into the reference sectors and 145 μL of sample was loaded into the sample sector in a 12-mm centerpiece. For higher concentration samples (>10 mg/mL), 50 μL of reference buffer was loaded into the reference sectors and 45 μL of sample was loaded into the sample sector in a 3-mm centerpiece. The sedimentation equilibrium experiments were performed at 5°C, at the specified speeds. Data were collected at 300 nm, every 0.001 cm in the step mode, with 20 averages per step. Equilibrium was confirmed using Winmatch and determined by the absence of deviations between successive scans.

To estimate the apparent molecular weight of each loading concentration of mAb-1,[19,20] the SE data were initially analyzed using an ideal, single-species sedimenting model by the program NONLIN,[21] according to the following equation:

$$A_{t,r} = A_{t,\mathrm{ref}} \cdot \exp\left(\frac{M_{b,\mathrm{app}} \cdot \omega^2}{RT} \cdot \frac{r^2 - r_{\mathrm{ref}}^2}{2}\right) + b \quad (1)$$

where $A_{t,r}$ is the total absorbance at radial position, r, $A_{t,\mathrm{ref}}$ is the total absorbance at the reference radial position, r_{ref}, ω is the angular velocity of the rotor, R is the gas constant, T is the absolute temperature, b is the baseline offset, and $M_{b,\mathrm{app}}$ is the apparent buoyant molecular weight given by