Opalescent Appearance of an IgG1 Antibody at High Concentrations and Its Relationship to Noncovalent Association

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Received December 18, 2003; accepted March 15, 2004

Purpose. Therapeutic antibodies are often formulated at a high concentration where they may have an opalescent appearance. The aim of this study is to understand the origin of this opalescence, especially its relationship to noncovalent association and physical stability.

Methods. The turbidity and the association state of an IgG1 antibody were investigated as a function of concentration and temperature using static and dynamic light scattering, nephelometric turbidity, and analytical ultracentrifugation.

Results. The antibody had increasingly opalescent appearance in the concentration range 5–50 mg/ml. The opalescence was greater at refrigerated temperature but was readily reversible upon warming to room temperature. Turbidity measured at 25°C was linear with concentration, as expected for Rayleigh scatter in the absence of association. In the concentration range 1–50 mg/ml, the weight average molecular weights were close to that expected for a monomer. Zimm plot analysis of the data yielded a negative second virial coefficient, indicative of attractive solute-solute interactions. The hydrodynamic diameter was independent of concentration and remained unchanged as a function of aging at room temperature.

Conclusions. The results indicate that opalescent appearance is not due to self-association but is a simple consequence of Rayleigh scatter. Opalescent appearance did not result in physical instability.

KEY WORDS: light scattering; protein aggregation; protein formulation; reversible association; turbidity.

INTRODUCTION

To accommodate relatively high therapeutic doses, antibodies are typically isolated and formulated at high (10–100 mg/ml) concentrations (1–5). Under these conditions, they often have an opalescent appearance (5). The apparent similarity of these opalescent solutions to aggregated protein solutions raises concerns with regard to irreversible association and its potential to cause immunogenicity or injection-site reactions (6), the feasibility of developing a stable high-concentration formulation, and the long-term physical stability of the bulk drug substance or formulated solutions (7). In an attempt to understand the origin of the opalescence of antibody solutions at high concentration, especially its relationship to noncovalent association, an IgG1 antibody (148.9 kDa) was investigated over a 100-fold concentration range (0.5–50 mg/ml). Solutions of this antibody had an increasingly opalescent appearance in the concentration range 5–50 mg/ml, which persisted even after filtration through a 0.2-μm sterilizing filter, suggesting that opalescent appearance was not caused by large filterable particles. It was also noted that this opalescence was greater at refrigerated temperatures (5°C) compared to that at room temperature. However, these temperature-dependent changes in the appearance were reversible. The studies described here were undertaken to address the following question: Is the opalescent appearance simply a consequence of high Rayleigh scatter expected of large molecules at high concentration (8) or is it a consequence of noncovalent association at high concentrations? Determination of the association state of the molecule directly at the high concentration is most relevant to assess its impact on the stability and biopharmaceutical properties; yet, it is complicated by the limitations of various instrumental techniques and the potential nonideality effects. For example, the high refractive index or absorbance at these high concentrations makes the application of analytical ultracentrifugation difficult (9–10). Similarly, the use of size exclusion chromatography is limited by the dilution and peak broadening that occurs, and the interaction of the antibody with the column material can potentially reverse the noncovalent association if it was originally present in the concentrated solution (11–12). Light scattering techniques on the other hand have a wider dynamic range with respect to concentration and allow direct measurement of association state of the molecule, avoiding any artifacts arising due to matrix interaction. However, one needs to be cautious about potential nonideality effects at high concentrations (8). Viscosity changes as a function of concentration can be quite significant for antibodies and need to be taken into consideration when calculating hydrodynamic diameter from dynamic light scattering data (13).

In order to understand the origin of the opalescent appearance and its relationship to noncovalent association, the antibody solutions were investigated as a function of concentration and temperature using static and dynamic light scattering and nephelometric turbidity. Analytical ultracentrifugation was used as an orthogonal technique to examine association state at low concentrations. Weight average molecular weights (WAMWs) were determined as a function of concentration using static light scattering (SLS), whereas hydrodynamic diameter was measured by dynamic light scattering (DLS). Reversibility of association was examined by measuring the WAMW and hydrodynamic diameter upon dilution into a surrogate physiological buffer, phosphate-buffered saline at pH 7.3. To understand the enhanced opalescent appearance at lower temperatures, concentration dependence of turbidity and hydrodynamic diameter were also measured at 5°C. The relationship of opalescence to physical stability was assessed by following the antibody solutions by DLS during a period of 12 days, both at room and refrigerated temperatures and as a function of concentration.

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ABBREVIATIONS: DLS, dynamic light scattering; EP, European Pharmacopoeia; IgG1, immunoglobulin type G1; IgG4, immunoglobulin type G4; MW, molecular weight; NTU, nephelometric turbidity unit; PBS, phosphate-buffered saline; SLS, static light scattering; WAMW, weight average molecular weight.

MATERIALS AND METHODS

Materials

IgG1 antibody, IgG4 antibody, trisodium citrate dihydrate, and sodium chloride were obtained from Eli Lilly and Company (Indianapolis, IN, USA). Dulbecco's phosphate-buffered saline (without calcium chloride and magnesium chloride) was obtained from Gibco (Carlsbad, CA, USA). Deionized water was used in making all solutions. Solutions were made by serial dilution of the IgG1 antibody at a concentration of 20 or 50 mg/ml into the corresponding buffer (10 mM citric acid, 100 mM NaCl, pH 5.5).

Turbidity Measurements

Turbidity of antibody solutions as a function of concentration was measured in 13-mm glass culture tubes at room temperature using a HACH 2100N turbidimeter (Hach Company, Loveland, CO, USA). The temperature dependence of turbidity was measured by following Rayleigh scatter on a Fluorolog-3 Fluorometer (JY Hoviba, Edison, NJ, USA) that allowed temperature control. Rayleigh scatter was measured at 510 nm, and the apparent turbidity was calculated by reference to a standard curve generated using suspensions of known turbidity.

Static Light Scattering

The weight average molecular weights of antibody solutions as a function of concentration were measured at 25°C using static light scattering at multiple angles (30–130°) on a Brookhaven Instruments Corporation (Holtsville, NY, USA) laser light scattering instrument. Samples were filtered through 0.22-μm, 13-mm Millex GV durapore membrane filters (Millipore, Billerica, MA, USA). The scatter data at 90° were used to derive WAMW. The complete data set at multiple angles was also used to create Zimm plots to derive molecular weight and second virial coefficient (8). For an ideal solution,

$$KC/R_\theta = 1/M \quad (1)$$

where, M is molecular weight, K is a constant, R_θ is Rayleigh ratio that combines a number of experimental parameters, and C is concentration.

The effects of solution nonideality can be incorporated in to an equation of the form,

$$KC/R_\theta = 1/M + 2BC + \cdots \quad (2)$$

where B is the second virial coefficient. To obtain M, it is necessary to extrapolate KC/R_θ to zero angle and zero concentration. A Zimm plot has KC/R_θ on the ordinate and $\sin^2(\theta/2) + kC$ on the abscissa, where k is an arbitrary constant, and allows both extrapolations to be made on the same graph. M and B can be obtained from the intercept and slope, respectively, of the zero angle line. The sign and magnitude of second virial coefficient are related to excluded volume effects and intermolecular interactions. Both excluded volume effects and intermolecular repulsive interactions result in a positive second virial coefficient, whereas attractive intermolecular interactions result in a negative second virial coefficient. For a given macromolecular solution, the balance of these various interactions determines the sign and magnitude of second virial coefficient.

Dynamic Light Scattering

The hydrodynamic diameter of antibody solutions as a function of concentration was determined at 25°C or 5°C by measuring the autocorrelation function at 90° scattering angle on a Brookhaven Instruments Corporation laser light scattering instrument. Samples were filtered through 0.22-μm, 13-mm Millex GV durapore membrane filters. The effective diameter and polydispersity was computed from the autocorrelation function using a quadratic fit. The intensity-based or volume-based distribution of diameter was determined using CONTIN analysis. Four separate measurements were made to derive average and standard deviation. The data were corrected for changes in viscosity of the antibody solutions as a function of concentration. The viscosity was measured at 5°C or 25°C using Viscolab4100 Laboratory Viscometer (Cambridge Applied Systems, Medford, MA, USA).

Equilibrium Sedimentation

Sedimentation equilibrium experiments were performed on a Beckman Model XLI ultracentrifuge (Fullerton, CA, USA). A 0.5 mg/ml solution of the antibody was loaded into a 2-sector, 0.297-cm path-length centerpiece cell with quartz windows and placed into an An-60 Ti 4-hole rotor. The cell was centrifuged at 8000 rpm, and radial scans at 280 nm were collected at 4-, 14-, 16-, and 18-h time points. The 16- and 18-h scans were superimposable, suggesting equilibrium had been achieved. The final data set is an average of five scans, and the error associated with absorbance measurements was in the range 0.001–0.006 AU. The 18-h radial scan was then fit to a single ideal species using the "self-association" model of the manufacturer-supplied version of Origin (XLI/XLA Data Analysis Software v. 4.0). Values of $\rho = 1$ g/ml and ν-bar = 0.73 ml/g were used in the calculation of molecular weight (14). In a sedimentation equilibrium experiment, a solution of the sample is centrifuged at a constant rotor velocity until equilibrium is reached. At equilibrium, a concentration gradient is established relative to the distance from the center of rotation that reflects the balance of sedimentation and diffusion. For a single solute component, the concentration gradient at equilibrium is related to molecular weight by

$$d \ln C/d(r^2) = \omega^2 M(1 - \nu\rho)/2RT \quad (3)$$

where C is the concentration at radius r (distance from the center of rotation), ω is the angular velocity, M is molecular weight, ν is the partial specific volume, and ρ is the solution density. Reversible macromolecular associations can be characterized quantitatively by using analogous expressions (9,15).

RESULTS AND DISCUSSION

Analytical Ultracentrifugation: Equilibrium Sedimentation

Equilibrium sedimentation analysis was carried out at a loading concentration of 0.5 mg/ml in citrate buffer (10 mM citric acid, 100 mM NaCl, pH 5.5), to establish the monomeric nature of the antibody at low concentrations (~0.2–1.0 mg/ml). The concentration gradient at equilibrium was analyzed to derive molecular weight. As shown in Fig. 1, the data fit very well to a monomer, yielding a molecular weight of 158058 Da.

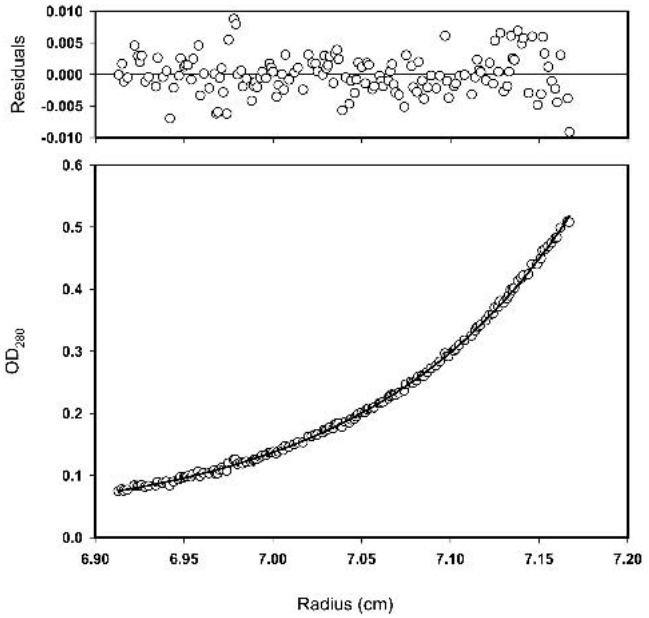

Fig. 1. Analytical ultracentrifugation equilibrium sedimentation analysis of IgG1 antibody at a loading concentration of 0.5 mg/ml. Radius plotted on abscissa is the distance from the center of rotation. Solid line indicates fit to a monomer. The residuals are plotted in the panel above the absorbance data.

Turbidity of IgG1 Solutions as a Function of Concentration

The turbidity of antibody solutions as a function of concentration is shown in Fig. 2. During the manufacture of the antibody, the final purified antibody was concentrated either to 20 mg/ml or 50 mg/ml. Starting from either of these stocks, solutions in the range 2.7–50 mg/ml were prepared by serial dilution. The results showed that at ambient temperature, the turbidity increased fairly linearly with concentration, from ~2 to 40 Nephelometric Turbidity Unit (NTU) in the concentration range 2.7–50 mg/ml. Based on European Pharmacopeia (EP) criteria (16), a clear solution is defined as one with turbidity less than or equal to reference suspension I, which has a turbidity of ~3 NTU. Based on this definition, only solutions less than ~5 mg/ml could be described as clear, and all concentrations greater than 5 mg/ml are opalescent. Nephelometric turbidity measurements detect Rayleigh scatter, which is expected to change linearly with concentration, in the absence of association or nonideality effects. The approximately linear change of turbidity with concentration initially suggested the absence of significant concentration-dependent association. As shown by analytical ultracentrifugation data, the molecule is monomeric at low concentrations. Thus, the high turbidity and opalescent appearance may be expected for antibodies at high concentrations, even in the absence of self-association, simply as a result of enhanced Rayleigh scatter due to high molecular weight and high concentration. This conclusion was further supported by SLS measurements that allowed determination of WAMWs as a function of concentration. Comparison of turbidity of antibody solutions derived from a 50 mg/ml bulk lot and a 20 mg/ml bulk lot showed that the turbidity vs. concentration plots overlap, suggesting that concentrating the antibody solutions up to 50 mg/ml did not cause any irreversible self-association.

Association State of Antibody as a Function of Concentration

The physical association state of the antibody was assessed by measuring weight average molecular weights as a function of concentration using static light scattering. The apparent weight average molecular weights of antibody solutions as a function of concentration, measured at 25°C, are shown in Fig. 3. The measured weight average molecular weights ranged from 0.9 to 1.3 times that expected for a monomer (~148.9 kDa), suggesting that association, if any, was not significant. At concentrations as high as 50 mg/ml, the effects of nonideality complicate a rigorous interpretation of weight average molecular weight data. Thus, the apparent increase in WAMW with concentration could either be due to small levels of association or nonideality effects. Interpretation of the data with either of these assumptions leads to the following conclusions.

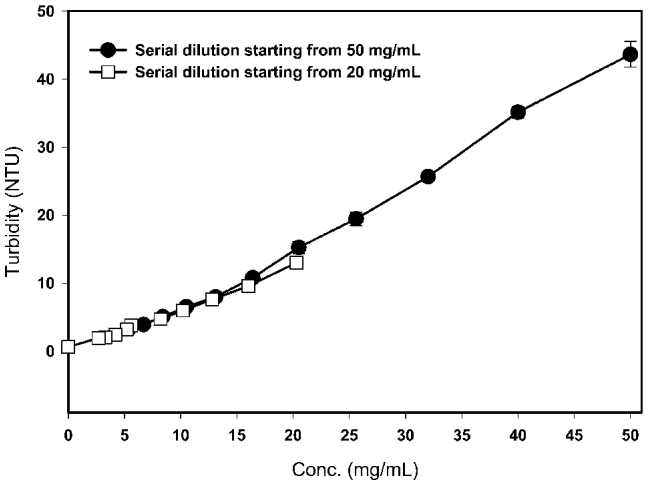

Fig. 2. Turbidity of antibody solutions measured as a function of concentration at ambient temperature using HACH turbidimeter. Filled circles correspond to serial dilution of a 50 mg/ml stock. Squares correspond to serial dilutions of a 20 mg/ml stock. Where error bars are not visible, they are smaller than the size of the symbol.

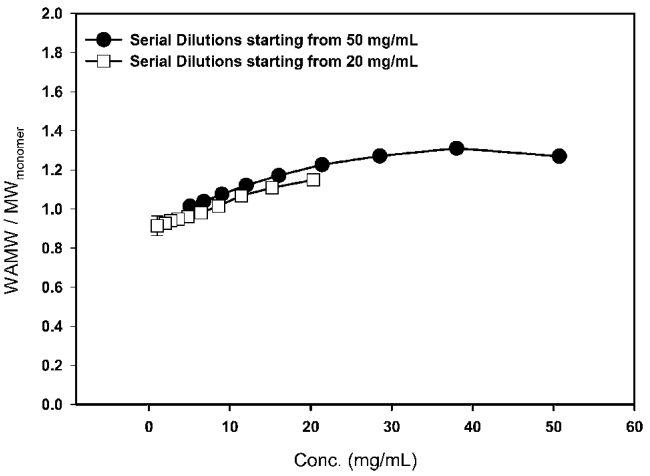

Fig. 3. Weight average molecular weight of antibody as a function of concentration, determined by static light scattering. Filled circles correspond to serial dilution of a 50 mg/ml stock. Squares correspond to serial dilutions of a 20 mg/ml stock. Where error bars are not visible, they are smaller than the size of the symbol.

Ignoring nonideality effects, the measured apparent weight average molecular weights would be consistent with, for example, a dimer content (mole fraction) of ~20% at 50 mg/ml. This analysis resulted in a K_D of ~0.8 mM for the monomer-dimer equilibrium, suggesting only a very weak interaction. If the data were interpreted as a monomer-oligomer (n = >3) equilibrium, the mole fraction of the oligomer would be correspondingly smaller (e.g., 2% of a pentamer at 50 mg/ml). Nonideality arising from attractive solute-solute interactions would also be expected to result in an apparent increase in the weight average molecular weights. Thus, Zimm plot analysis of the angle and concentration-dependent static light scattering data resulted in a molecular weight of 153 kDa, very close to that of a monomer, and yielded a negative second virial coefficient of -1.61×10^{-5} cm^3 mol/g^2, consistent with attractive solute-solute interactions. However, note that a negative virial coefficient is indistinguishable from association. For a spherical molecule, excluded volume effects alone would have resulted in a value of approximately 3×10^{-5} cm^3 mol/g^2 for the second virial coefficient (8) and a decrease in apparent WAMW with increasing concentration. Therefore, attractive solute-solute interactions and/or association in this case have more than compensated the excluded volume effects. The expected turbidity for a solution composed entirely of a monomer, and subject to excluded volume effects, could be calculated assuming a second virial coefficient of 3×10^{-5} cm^3 mol/g^2 due to excluded volume effects and, in addition, accounting for the observed negative second virial coefficent. These calculations would result in a corrected turbidity of 3.0 NTUs at 5.0 mg/ml and 28.5 NTUs at 50 mg/ml. Thus, notwithstanding the slight increase in WAMW as a function of concentration, considering SLS data together with turbidimetry, it is clear that in the case of this antibody, solutions composed entirely of a monomer would still appear opalescent at concentrations >5 mg/ml.

Comparing the WAMWs of solutions derived from a 50 mg/ml lot and a 20 mg/ml lot, it was seen that they were very similar at equivalent concentrations, suggesting, as with turbidity measurements, that concentrating the bulk drug substance to 50 mg/ml did not cause significant irreversible self-association.

Hydrodynamic Diameter of Antibody as a Function of Concentration

The physical association state of the antibody as a function of concentration was also assessed by measuring mean hydrodynamic diameter using dynamic light scattering, a technique that also allows sensitive detection of large aggregates. The apparent mean hydrodynamic diameter of the antibody, measured at an antibody concentration of 0.5 mg/ml, was 10.1 ± 0.6 nm, close to that expected for a hydrated sphere with a molecular weight of ~150 kDa. A value of 8 nm was calculated using a hydration of 0.37 g of water/g of dry protein and the expression

$$R_0 = [3M(v + \delta v_0)/4\pi N]^{1/3} \quad (4)$$

where M is the molecular weight, v is specific volume, v_0 is the specific volume of solvent, and N is Avogadro's number. The polydispersity index calculated based on cumulant analysis was 0.07 ± 0.02. The apparent mean diameter increased with concentration as shown in Fig. 4. However, the mean diameter measured by dynamic light scattering is derived from the diffusion coefficient, which depends on the viscosity. Stokes-Einstein equation relates the limiting diffusion coefficient to the hydrodynamic diameter (8),

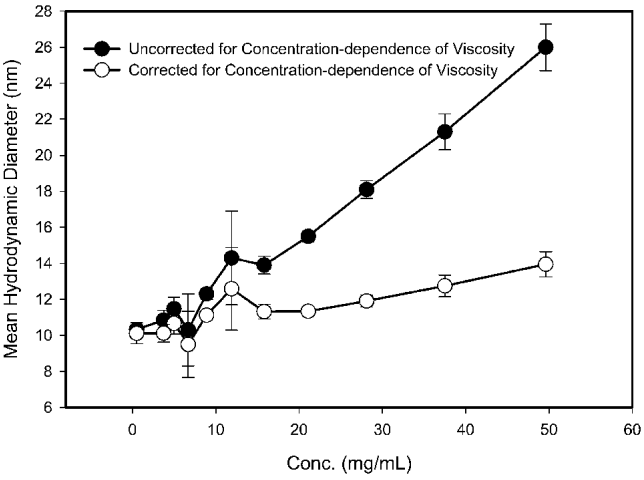

Fig. 4. Hydrodynamic diameter of antibody solutions as a function of concentration, measured at 25°C. Filled circles represent apparent hydrodynamic diameter uncorrected for concentration-dependent changes in the viscosity of the solutions. Open circles represent data corrected using measured viscosity. Where error bars are not visible, they are smaller than the size of the symbol.

$$D_0 = k_B T/3\pi\eta d_h \quad (5)$$

where D_0 is the diffusion coefficient, k_B is Boltzmann's constant, T is the absolute temperature, η is the bulk viscosity of the liquid in which the particle moves, and d_h is the spherical equivalent hydrodynamic diameter. As shown in Fig. 4, the hydrodynamic diameter corrected for viscosity was fairly independent of concentration. The differences as a function of concentration were within the variability of this method (~±2.0 nm).

In addition to the viscosity effects, the nonideality effects can also play a role at high concentrations. For example, in dilute solutions, when the distance between the scatterers is large compared to q^{-1}, self-diffusion is measured, and when the distance between the scatterers is small compared to q^{-1}, mutual-diffusion is measured (q is the magnitude of scattering wave vector, given by $4\pi\eta/\lambda_0 \sin(\theta/2)$, where λ_0 is the wavelength of laser in vacuum and θ is the scattering angle). Mutual- and self-diffusion vary differently with concentration, depending on the attractive and repulsive forces involved. In dilute solutions, both self- and mutual-diffusion asymptotically approach the same limit D_0, given by Eq. (1) above.

The approximate dependence of diffusion coefficient of a spherical protein on nonideality and viscosity can be described as (8),

$$D_0 = RT[1 + 2BMc]/6\pi N\eta R_0 \quad (6)$$

where D_0 is the diffusion coefficient, R is the gas constant, T is the absolute temperature, B is the second virial coefficient, M is the molecular weight, c is the concentration in g/ml, N is Avogadro's number, η is the viscosity of the liquid, and R_0 is the radius of the particle. This leads to a correction factor of $(1 + 2BMc)/(\eta/\eta_0)$ for the radius, where η_0 is the viscosity of the solvent. Assuming only excluded volume effects, B =

4v/M, where v is the specific volume. Using $(\eta/\eta_0) = 1.88$, based on measured viscosity at 50 mg/ml, and a specific volume of 0.73 ml/g, this equation results in a correction factor of ~0.7 to the measured diameter at a 50 mg/ml concentration, based on excluded volume effects alone. If solute-solute attractive interactions are invoked, for example, a value of -2×10^{-5} cm^3 mol/g^2 for B, a correction of ~0.5, is obtained.

Though it is not possible to determine accurately the applicable correction factor, the above analysis indicates that the combined effects of viscosity and nonideality can reasonably result in as much as a 50% correction at 50 mg/ml. Therefore, the apparent differences in measured mean diameter as a function of concentration should be interpreted with caution and may not readily indicate concentration-dependent association. In conjunction with the static light scattering results described above, it is reasonable to conclude that association, if any, is not significant and is readily reversible upon dilution. In addition, the DLS data were consistent with a single distribution of hydrodynamic size devoid of large aggregates, even at an antibody concentration of 50 mg/ml.

Reversibility of Physical Association of Antibody

The static and dynamic light scattering measurements described above indicate a modest increase in apparent WAMW and hydrodynamic diameter with concentration. Though these increases are not significant and may not be readily ascribed to self-association, the reversibility of these effects upon dilution was examined.

One of the concerns raised by the opalescent appearance of antibody solutions at high concentrations is the potential for physical aggregation that may cause tissue reactions at the subcutaneous injection site. However, substantial dilutions [e.g., tissue blood flow of the order of 1 ml/10 g. min (17)] are expected at the subcutaneous site. Therefore, in assessing the potential impact of association that is present at formulated concentrations, it is pertinent to consider reversibility upon dilution. Insulin, for example, is present as a hexamer in formulated solutions and dissociates into monomers upon dilution at the subcutaneous site before getting absorbed (18–20).

In order to establish that the apparent modest increase in WAMW and hydrodynamic diameter at the high concentration were reversible upon dilution, the 50 mg/ml antibody solution in citrate buffer at pH 5.5 was diluted into a surrogate physiological buffer, phosphate-buffered saline (PBS) at pH 7.3, and the resulting solutions were examined by static and dynamic light scattering. Table I lists WAMW and hydrodynamic diameter of antibody in solutions obtained by dilution in to PBS. It was seen that upon 10- and 100-fold dilution into PBS, the resulting WAMW or hydrodynamic diameter were close to those expected for a monomeric antibody and were comparable to those in citrate buffer.

Temperature Dependence of Opalescence

Figure 5 shows the temperature dependence of turbidity of antibody solutions measured by following Rayleigh scatter on a spectrofluorometer. Again, it was noted that at 25°C and 37°C, the measured turbidities were linear with concentration. The turbidity measured at 5°C, however, was nonlinear with concentration, with an upward curvature at higher concentrations. This enhanced turbidity at lower temperature and higher concentrations is likely a result of reversible noncovalent self-association. To examine further the association state of the molecule at 5°C, hydrodynamic diameter was measured by DLS at this temperature, and the data were corrected for changes in viscosity. Figure 6 shows the hydrodynamic diameter as a function of concentration. The hydrodynamic diameter appears to increase with concentration, indicating that the enhanced opalescent appearance at high concentrations (>20 mg/ml) at this temperature may be a result of noncovalent association. The effects seen at 5°C were reversed when the temperature was increased to 25°C or 37°C. Alternatively, the enhanced opalescent appearance at 5°C may indicate approach to a liquid-liquid phase-separation temperature, which is expected to be accompanied by enhanced light scattering (21–22). This potential phase behavior needs further investigation.

Physical Stability of Antibody Solutions at Room and Refrigerated Temperatures

In order to assess potential impact of opalescence on physical stability, the mean hydrodynamic diameter of antibody solutions in the concentration range 0.5–50 mg/ml was followed as a function of aging at room temperature for 12 days. These data are shown in Fig. 7. The solutions were filtered only once, prior to the first measurement, and were allowed to age in the glass tubes intended for DLS measurement, thus avoiding the removal of any aggregates that might have grown upon aging. The DLS measurements were made at 25°C. The mean hydrodynamic diameter was virtually unchanged at any of these concentrations, suggesting high degree of physical stability even at concentrations as high as 50 mg/ml. The hydrodynamic diameter of a 50 mg/ml solution was also measured as a function of aging at refrigerated temperature for 1 week and remained unchanged (12.3 vs. 13.2 nm). This limited evaluation of physical stability suggests that opalescent appearance does not result in growth of soluble aggregates or precipitation during a reasonable period of time. This degree of physical stability will allow development

Table I. Molecular Size and Association Properties of Antibody Solutions in Phosphate-Buffered Saline, Determined by Light Scattering

Fold dilution	Conc. (mg/ml)	WAMW (kDa) (PBS)	WAMW (kDa) (citrate buffer)	Diameter (nm) (PBS)	Diameter (nm) (citrate buffer)
No dilution	50.7	*	189	*	12.5
10	5.07	153	151	11.8	11.3
100	0.507	140	*	10.9	10.8

PBS, phosphate-buffered saline; WAMW, weight average molecular weight.
* Data not collected.

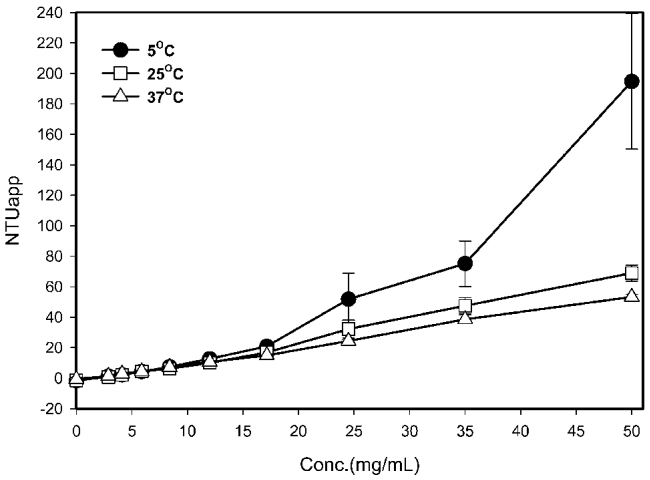

Fig. 5. Temperature and concentration dependence of turbidity of antibody solutions measured by monitoring Rayleigh scatter on a fluorometer. Circles, squares, and triangles represent data at 5, 25, and 37°C, respectively. Error bars represent data from two separate experiments using two different lots of material. Where error bars are not visible, they are smaller than the size of the symbol.

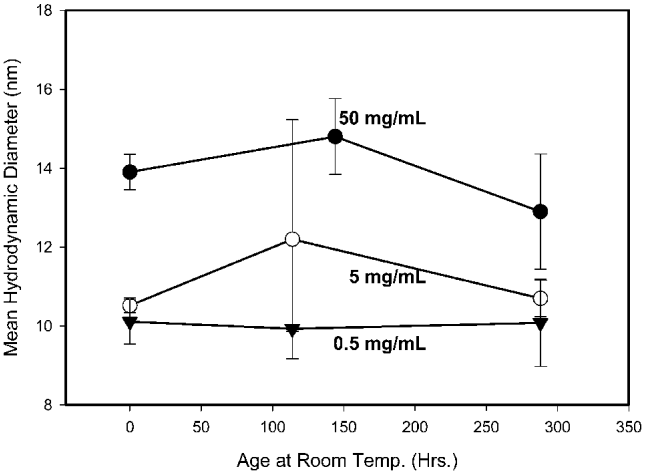

Fig. 7. Physical stability of antibody solutions as a function of aging at room temperature. Data at 0.5, 5, and 50 mg/ml are shown. The data are corrected for the measured viscosity of the solutions.

of a lyophilized formulation, where reconstituted solutions need only be exposed to room or refrigerated temperatures for brief periods of time. Though further work is needed to establish long-term solution stability, the detailed characterization of association of the antibody molecule presented here suggests that opalescent appearance may not represent a significant risk factor in the development of such a formulation.

CONCLUSIONS

The opalescent appearance of the IgG1 antibody investigated here appears to be unrelated to noncovalent association. At low concentrations, the weight average molecular weight is consistent with that expected for a monomer, and over the concentration range of 1–50 mg/ml, the weight average molecular weight did not change significantly (0.9x – 1.3x, where x is monomer molecular weight). This slight concentration dependence of the weight average molecular weights is consistent with either weak monomer-dimer equilibrium or a negative virial coefficient, indicative of attractive solute-solute interactions. These data, together with nephelometric turbidity measurements, indicate that even in the absence of association, antibody solutions begin to appear opalescent at high concentrations (greater than approximately 5 mg/ml), due to high Rayleigh scatter as a result of their large molecular weight. It is important to note, however, that all opalescence in concentrated antibody solutions cannot be attributed to Rayleigh scatter from monomeric species. The relative opalescent appearance of solutions of different antibody molecules at a given concentration may be somewhat different, depending on the nature of solute-solute interactions. Some antibodies may also be characterized by reversible or irreversible association, which is expected to increase further the opalescent appearance. In addition to association, the extent of opalescence will be dictated by the direction of nonideality, that is, decreasing or increasing normalized Rayleigh scatter as a function of concentration, due to positive or negative virial coefficients, respectively. Excluded volume effects and repulsive intermolecular interactions will result in a positive virial coefficient, whereas attractive intermolecular interactions will result in a negative virial coefficient. Different classes of antibodies or different antibodies in the same class may have very different types of interactions. Interestingly however, when we examined the nephelometric turbidity of another antibody that belongs to an IgG4 class as a function of concentration, this antibody also exhibited approximately linear dependence of turbidity as a function of concentration (data not shown), resulting in 18.4 NTUs at 26 mg/ml, similar to that of the IgG1 antibody studied here. The enhanced opalescent appearance at 5°C observed here may be related to reversible self-association or approach to a liquid-liquid phase separation temperature. At room temperature, the mean hydrodynamic diameter measured by DLS was consistent with that expected for a monomeric antibody and confirmed the absence of large molecular aggregates. However, at high concentrations, changes in the viscosity of the

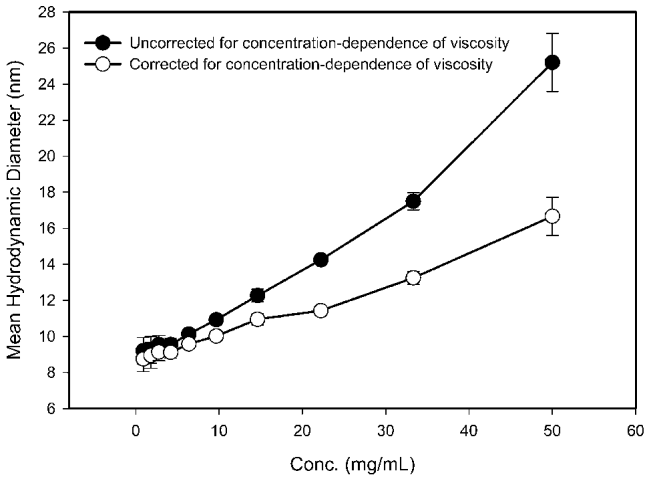

Fig. 6. Hydrodynamic diameter of antibody solutions as a function of concentration, measured at 5°C. Filled circles represent apparent hydrodynamic diameter uncorrected for concentration-dependent changes in the viscosity of the solutions. Open circles represent data corrected using measured viscosity. Where error bars are not visible, they are smaller than the size of the symbol.

solutions can result in significant increase in the apparent diameter. Therefore, with antibody therapeutics, it is important to correct the hydrodynamic diameter for changes in viscosity. The apparent modest changes in weight average molecular weight and hydrodynamic diameter were readily reversed upon dilution into a physiological buffer. Thus, despite the ambiguities that result from nonideality effects at high concentration, reversibility can be established with a great degree of certainty and should be evaluated in considering potential impact of molecular association on injection-site reactions and immunogenicity (6).

ACKNOWLEDGMENTS

We thank Drs. Mark Brader, Brian Fahie, Henry Havel and Uma Kuchibhotla for critical reading of the manuscript and their comments, Dr. Thomas Furman for purified antibody preparations and Dr. Jirong Lu for early discussions on opalescent appearance.

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