

# Reversible Self-Association Increases the Viscosity of a Concentrated Monoclonal Antibody in Aqueous Solution

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**ABSTRACT:** This study was conducted to investigate the effect of reversible protein self-association on the viscosity of concentrated monoclonal antibody solutions. The viscosities of the monoclonal antibody solutions were measured by either a capillary viscometer or a cone-plate rheometer at different protein concentrations, pH, and ionic strength. Soluble aggregates were determined by size exclusion chromatography, light scattering, and analytical ultracentrifugation. Self-association of protein at high protein concentration was monitored by sedimentation equilibrium analysis using a preparative ultracentrifuge and a microfractionator. The viscosity of one of the monoclonal antibodies investigated is highly dependent on protein concentration, pH, and ionic strength of buffer and charged excipients. This antibody shows the highest viscosity near its pI at low ionic strength conditions. Sedimentation equilibrium analysis suggests that this antibody tends to reversibly self-associate at high protein concentration. The self-association appears to be quite weak and is not detectable by sedimentation velocity and size exclusion chromatography at low protein concentration. There are no significant differences in the amounts of non-dissociable soluble aggregates formed between low viscosity and high viscosity samples. These results suggest that the reversible multivalent self-association of this protein appears to be mediated mainly by electrostatic interactions of charged residues and results in unusually high viscosity of this monoclonal antibody in solution at low ionic strength conditions. © 2005 Wiley-Liss, Inc. and the American Pharmacists Association *J Pharm Sci* 94:1928–1940, 2005

**Keywords:** monoclonal antibody; high protein concentration; viscosity; self-association; sedimentation equilibrium; sedimentation velocity

## INTRODUCTION

Over the last decade, monoclonal antibody therapy has evolved and become an important class of drugs for treating numerous human diseases, including cancer, allergic diseases, asthma, and organ transplantation.<sup>1–3</sup> Approximately 25% of protein drugs currently under development are full length, fragment, or conjugated versions of monoclonal antibodies (<http://www.phrma.org>).

Since antibodies are part of the natural human defense systems that are capable of interacting with virtually any antigen molecules at high affinity and specificity, they are ideal candidates for targeted therapy. By the end of 2003, fourteen therapeutic antibodies, antibody fragments, or conjugated antibodies had been licensed by the US Food and Drug Administration (FDA) to treat a wide range of human diseases.<sup>4</sup>

Monoclonal antibody therapy is often given on a regular basis and requires several mg/kg dosing by injection.<sup>5</sup> The majority of approved monoclonal antibody therapeutics, especially for oncology indications is administered by the IV route.<sup>6</sup> However, several ongoing monoclonal antibody development

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programs are targeting diseases that may require outpatient administration or home use, and hence may necessitate the development of alternate delivery routes. Oral, transdermal, and pulmonary routes of administration have been difficult to achieve because of the instability and sizes of monoclonal antibodies. For example, one monoclonal antibody given by the pulmonary route had low systemic distribution and was clinically ineffective.<sup>7</sup> Subcutaneous (SC) administration is the preferred route used in a physician's office/clinic or by the patient at home. SC administration is generally limited to small injection volume, and for the high dose antibody therapy, it often necessitates the development of high protein concentration formulations. For example, if a protein is to be administered to a patient at 2 mg/kg on a weekly basis, the average weekly dose will be 130 mg considering 65 kg as the average body weight for patients. Since an injection volume of more than 1.5 mL is not well tolerated for SC administration, the protein concentration for weekly SC administration would have to be approximately 100 mg/mL (130 mg protein in less than 1.5 mL volume). Because protein molecules are large and complex, development of protein formulations at such high concentration poses many challenges. These include not only maintaining the physical and chemical stability throughout the shelf-life, but also having physical properties that are compatible with manufacturing, storage, and delivery.<sup>8</sup> Concentration dependent protein properties such as viscosity can have a major impact on the manufacturing process, especially tangential flow filtration (TFF) techniques used to concentrate and formulate the protein.<sup>8</sup> In particular, high viscosities may result in pressure drops during TFF that exceed the pump performance specifications. High viscosity of a protein formulation can also make it more difficult to administer the protein drug by injection, particularly for SC delivery, where delivery of high viscosity of solution within a reasonable time frame requires the use of larger bore needles, which may result in more painful SC injections. Therefore, it is important to optimize the formulation resulting in a reduced viscosity that is well suited for manufacturing and administration.

Most protein molecules are only marginally stable in solution. The structure and conformation of native protein can be altered under stressed conditions and lead to formation of irreversible soluble aggregates or particulates. Although molar concentration of osmolytes, such as sugars,

have been used to prevent protein aggregation from nonnative protein in solution,<sup>9,10</sup> this apparent fix is limited since it may also add to the viscosity and osmolality of the formulation that may render it impractical for use as a SC delivery. In addition, high concentration of sugar can enhance the reversible self-association of native protein,<sup>11–13</sup> particularly at high protein concentration, in which the weak reversible self-association can increase significantly. This can have a major impact on the physical properties of protein formulations. Due to these challenges, to date, very few marketed therapeutic monoclonal antibodies have been successfully formulated as liquids above 100 mg/mL.

Reversible self-association of a human myeloma protein has been shown to increase viscosity through a combination of weak nonionic or hydrophobic interactions.<sup>14</sup> In addition, self-association of monoclonal serum immunoglobulins has also been associated with the hyperviscosity syndromes of various disorders.<sup>15–17</sup> The present study seeks to understand the effects and contributions of a reversible protein self-association on the viscosity of a concentrated monoclonal antibody solution. These studies led to the development of an improved formulation with reduced viscosity that was well suited for SC delivery.

## MATERIALS AND METHODS

### Materials

Three humanized monoclonal antibodies with different complementary determining regions (CDRs), MAb1, 2, and 3 were constructed from the same IgG1 human framework with  $\kappa$  light chain. These antibodies were expressed in Chinese hamster ovary (CHO) cell lines, and prepared by a series of chromatography methods including affinity purification by protein A chromatography and ion exchange chromatography. The antibodies were prepared as lyophilized materials or as solutions using TFF processes. All the chemical reagents were analytical grade or higher. The actual compositions of the solutions are discussed in the appropriate "Results" section.

### Analytical Methods

#### Size Exclusion Chromatography

Size exclusion chromatography experiments were conducted using a TosoHaas TSK Super SW 3000

(4.6 × 300 mm) column on an HP 1100 HPLC system (Agilent Technologies, Palo Alto, CA). The column was loaded with ~30 µL of protein at ~1, 125, or 150 mg/mL. The samples were eluted at ambient temperature with 0.1M potassium phosphate, 0.1M sodium chloride, and pH 6.8 with an isocratic flow rate of 0.2 mL/min. The elution peaks were monitored at 280 nm by a UV diode array detector (Agilent Technologies).

### Viscosity Measurement

The viscosity of a protein sample was initially measured using a Cannon-Fenske Routine viscometer (Cannon Instrument, State College, PA). Approximately 8 mL of solution was added to a capillary viscometer of size 50 for liquid samples with kinematic viscosity ranging from 0.8 to 4 cSt or size 200 for those ranging from 20 to 100 cSt. The viscometer was placed into a holder vertically and inserted into a MGW Lauda water bath (Brinkmann Instrument, Westbury, NY) that was maintained at 25°C. After the solution reached thermal equilibrium with the water bath, the solution was drawn above the upper fiducial mark. The efflux time was measured by allowing the liquid sample to flow freely down past the mark. To obtain kinematic viscosity in mm<sup>2</sup>/s (cSt), efflux time was multiplied by the viscometer constant of 0.004 for size 50 and 0.1 for size 200.

To evaluate the shear rate effect over a range of 0.1–10000/s, the viscosities of samples in units of mPaS were measured with a MCR300 rheometer (Anton PAAR, Ashland, VA) using a CP50-1 cone/plate measuring system. Approximately 0.6 mL of samples were loaded onto the lower measuring plate and allowed to come to thermal equilibrium at 25°C. Excess sample was removed by a pipette once the gap has been set. A solvent trap was used to prevent solvent evaporation.

The measured viscosity was obtained in units of mPas that can be related to the kinematic viscosity by dividing the value by the solution density.

### Concentration Determination

The concentration of antibody was obtained using a Hewlett Packard 8453 diode array spectrophotometer with a 1-cm quartz cuvette. Approximately 10 mg of neat samples were weighed and transfer into a volumetric flask, and then diluted with H<sub>2</sub>O to ~0.5 mg/mL. The dilution factor was determined using a density of 1.05 g/L for neat sample measured using a DMA35 digital densitometer (Parr, Graz, Austria). The UV absorbance at

280 nm was corrected for light scattering by subtracting the absorbance at 320 nm. This approximation for low levels of light scattering (<0.005) yields essentially the same correction when using the extrapolation method of Englander and Epstein.<sup>18</sup> The concentration was calculated using an absorptivity of 1.5/cm/(mg/mL) as determined by quantitative amino acid analysis.

### Sedimentation Velocity Studies

Sedimentation velocity experiments were conducted in a Beckman XL-I analytical ultracentrifuge. Samples were diluted to 1 mg/mL in PBS (0.1M potassium phosphate, 0.1M sodium chloride, pH 6.8), and then filled into a cell equipped with a 2 sector charcoal filled Epon centerpiece. Centrifugation was performed at 60000 rpm and 10°C, and the data were collected using the UV-absorption optical scanning system. The distribution of the different species was obtained using the non-linear curve-fitting program SEDFIT.<sup>19</sup>

### Sedimentation Equilibrium Studies

Sedimentation equilibrium analyses of antibodies at high protein concentration were conducted in a Beckman XL-I ultracentrifuge, using a preparative method described previously.<sup>20</sup> Approximately 300 µL of protein solution were layered on top of 100 µL of Fluorocarbon (FC-43) in thick-walled polycarbonate tubes. The samples were then centrifuged at 15000 rpm in a swinging-bucket SW 60 Ti rotor for 36–48 h. Attainment of sedimentation equilibrium was verified by comparing data obtained after centrifugation for 36 and 48 h. Immediately after centrifugation, the contents of each tube were collected in 10 µL fractions using a BRANDEL<sup>®</sup> automated micro-fractionator (minimum step size: 5 µm). The collected fractions were placed into a 96-well UV plate and diluted with PBS for measuring protein concentration by UV absorption spectroscopy.

The apparent weight average molecular weight,  $M_{w,app}$  at each radial position was determined with the following equation:

$$c(r) = c_0 \exp \left[ \frac{M_{w,app}(1 - \bar{v}\rho)\omega^2}{RT} \xi \right] \quad (1)$$

where  $c(r)$  is the protein concentration at radial position  $r$ ,  $c_0$  is the initial loading protein concentration,  $\bar{v}$  is the partial specific volume,  $\rho$  is the buffer density,  $\omega$  is the angular velocity,  $\xi = (r^2 - r_0^2)/2$ , and  $r_0$  is the reference radial position. Values of  $\bar{v}$  for MAb1 (0.73 mL/g), MAb2

(0.72 mL/g), and MAb3 (0.73 mL/g) were calculated from their amino acid and carbohydrate compositions.<sup>21,22</sup> The buffer density,  $\rho$  (1.01 g/mL) was determined using a Parr DMA35 digital densitometer (Parr).

The non-ideality of a protein solution is described by the second virial coefficient,  $B$  using the following equation<sup>23,24</sup>:

$$\frac{1}{M_{w,app}} = \frac{1}{M_w} + 2BC_p \quad (2)$$

where  $M_{w,app}$  is the apparent weight average molecular weight,  $M_w$  is the weight average molecular weight,  $B$  is the second virial coefficient, and  $C_p$  is the protein concentration.

## RESULTS AND DISCUSSION

### Viscosity of Protein Solution

The viscosity of MAb1 was determined over a concentration range from 30 to 125 mg/mL at 25°C. The samples were prepared by diluting a reconstituted lyophilized preparation with a buffered solution yielding samples with protein concentrations ranging from 30 to 125 mg/mL in 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20 at pH 6.0. The viscosities of the reconstituted samples were measured using a MCR300 rheometer. The viscosity of MAb1 is highly dependent on the protein concentration and increases nonlinearly with increasing antibody concentration. At 25°C, MAb1 at 125 mg/mL is about 60-fold more viscous than the solution without protein and impacts the time to draw the solution up into a syringe equipped with a 27 g needle (Figure 1).

The viscosity of MAb1 at high protein concentration is also dependent on the shear rate, and decreases with an increase of shear rate (Figure 2). This indicates that the concentrated MAb1 under these conditions behaves as a non-Newtonian fluid. Since non-Newtonian fluids often contain several micro structural species that are made of large and elongated aggregates, rather than smaller uniform non-interacting monomers, this result suggests that MAb1 may undergo self-association. The shear rate dependence is only observed at high protein concentration but not at low protein concentration (Figure 2). This observation suggests that these micro-structural aggregates are also fully reversible, and only form at higher protein concentration.

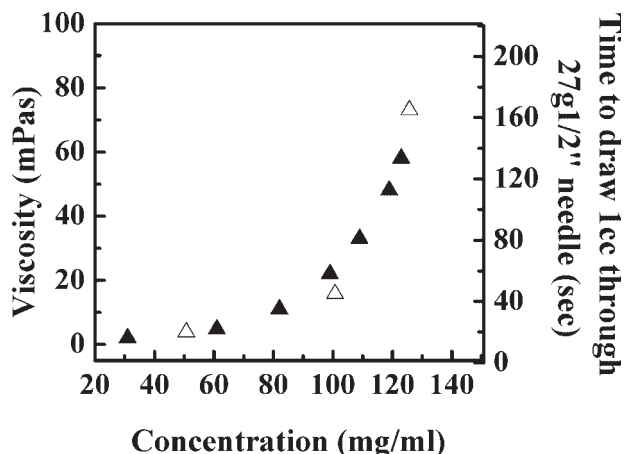


**Figure 1.** The effects of protein concentration on viscosity ( $\blacktriangle$ ) and syringability ( $\triangle$ ) of reconstituted MAb1. The samples contain 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20, pH 6.0. The viscosity was measured with a MCR300 rheometer (Anton PAAR) using a CP50-1 cone/plate measuring system at 25°C at a shear rate of 200/s. Time to draw solution into a syringe, syringability, was measured by drawing the plunger to the end of a 1 cc syringe barrel.

### Viscosity as a Function of Ionic Strength and pH

The viscosity of MAb1 is also dependent on the concentration of NaCl. Figure 3 shows the result of a MAb1 at 125 mg/mL in 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20 at pH 6 as

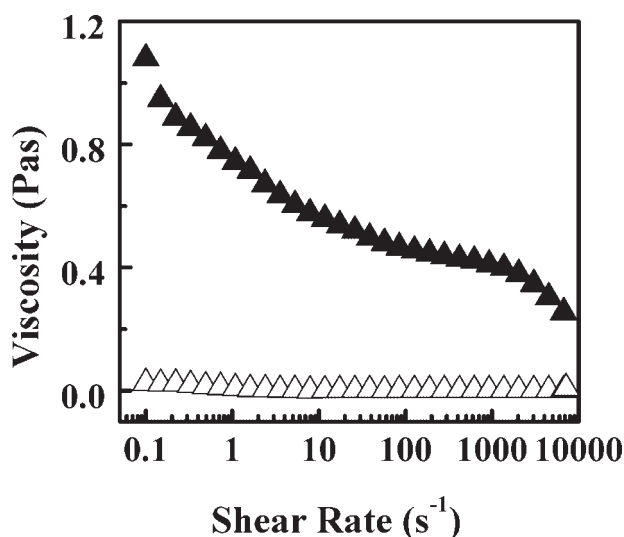


**Figure 2.** The effects of shear rate on viscosity of MAb1 at 10 mg/mL ( $\triangle$ ) and 200 mg/mL ( $\blacktriangle$ ). The sample contains 425 mM sucrose, 25 mM histidine, and 0.05% polysorbate 20 at pH 6.0. The viscosity was measured with a MCR300 rheometer (Anton PAAR) using a CP50-1 cone/plate measuring system at 25°C.

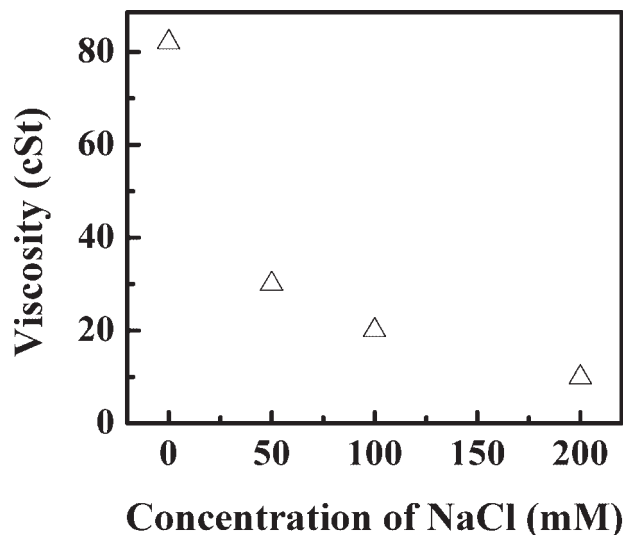


**Figure 3.** The effects of NaCl concentration on the kinematic viscosity of reconstituted MAb1. The samples contain 125 mg/mL of MAb1, 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20 and various amounts of NaCl at pH 6. The viscosity was measured using a Cannon-Fenske Routine viscometer (Cannon Instrument) at 25°C.

a function of NaCl concentration. The kinematic viscosity was determined at 25°C using a Cannon-Fenske Routine capillary viscometer. As the concentration of NaCl increases, the viscosity of the MAb1 decreases significantly. At 100 mM NaCl, the viscosity of MAb1 is about fourfold less than the solution in the absence of NaCl. The viscosity of this monoclonal antibody solution appears to begin to level off as the concentration of NaCl approaches 200 mM. The addition of NaCl does not appear to have any significant impact on conformation of MAb1. Both far UV and near UV CD spectrum (data not shown) clearly indicate that MAb1 has essentially the same conformation in solution with or without 150 mM NaCl.

The effects of different salts on the viscosity of MAb1 solutions were also investigated. Solutions of MAb1 at 40 mg/mL protein in 12 mM histidine, 255 mM sucrose, and 0.01% polysorbate 20 at pH 6 were prepared. The concentrated salt solutions (~3 M) were then added into solution to adjust the final salt concentration from 0 to 200 mM, resulting in <7% change in total protein concentration. The viscosity of the antibody solution decreases with increasing salt concentration and is clearly dependent on the final ionic strength of the solution (Figure 4). Most salts tested appear to have a similar effect on viscosity of samples. The change of viscosity does not appear to follow the Hofme-

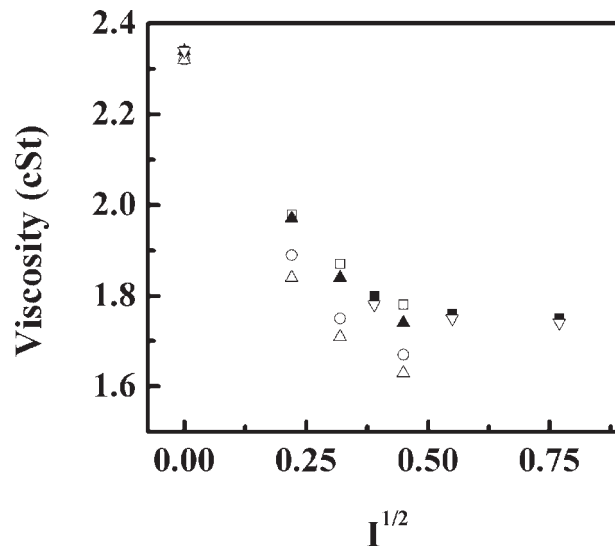


**Figure 4.** The effects of various salts as a function of ionic strength (plotted as  $I^{1/2}$ ) on the kinematic viscosity of MAb1 solution containing 40 mg/mL of protein, 10 mM histidine, 250 mM sucrose, 0.01% polysorbate 20 and various amounts of salts at pH 6. The viscosity was measured using a Cannon-Fenske Routine viscometer (Cannon Instrument) at 25°C. The salts included NaCl (□), NaSCN (○), NH<sub>4</sub>SCN (△), (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> (■), NH<sub>4</sub>Cl (▲), and CaCl<sub>2</sub> (▽).

ister series.<sup>25</sup> However, salts containing thiocyanate reduce viscosity more significantly than other salts. This observation may be related to the chaotropic nature of thiocyanate ions that results in a more efficient decrease of protein intermolecular interactions. Further work will be required to elucidate the role of particular ions such as thiocyanate in reducing the concentration dependence of the viscosity. Overall, these results strongly suggest that electrostatic interactions play a major role in causing the high viscosity of the MAb1 solution.

The effects of pH on the viscosity of a concentrated MAb1 solution, was examined in buffers that contain low and high salt concentration. The pH of the samples were adjusted by adding small amounts of 10% acetic acid or 0.5M arginine into an unbuffered MAb1 solution that had been concentrated to ~130 mg/mL in Milli-Q water. The final concentrations of total buffer and salt under "low salt conditions" were maintained at ~17.5 mM. The high salt samples were produced by adding a small volume of 5 M NaCl resulting in a final NaCl concentration of ~150 mM. The viscosity of the solution was determined at 25°C using a Cannon-Fenske routine capillary viscometer to obtain kinematic viscosities in cSt.

The viscosity of the MAb1 solution is highly dependent on the pH of the buffer, especially under low salt conditions (Figure 5). At near neutral pH, where it approaches the theoretical pI of the protein, the maximum value of the viscosity was observed at low salt condition. Moreover, the viscosity decreased significantly as a function of ionic strength. These results strongly suggest that electrostatic interactions contribute to the observed high viscosities. At the extreme pH values where the protein has a high net charge, electrostatic repulsive interactions will be maximized. However, MAb1 viscosity at the extreme pH values is not highly dependent on ionic strength and is at minimum values suggesting that repulsive electrostatic interactions do not contribute significantly to the high viscosities. Thus, these observations support the hypothesis that electrostatic attractive interactions are responsible for the high viscosity of this antibody in solution.

#### Viscosity Is Independent of the Presence of Non-Dissociable Soluble Aggregates

To determine if non-dissociable soluble aggregates account for the observed high viscosity of MAb1 in solution, size exclusion chromatography and sedimentation velocity analytical ultracentrifugation were used to determine presence of

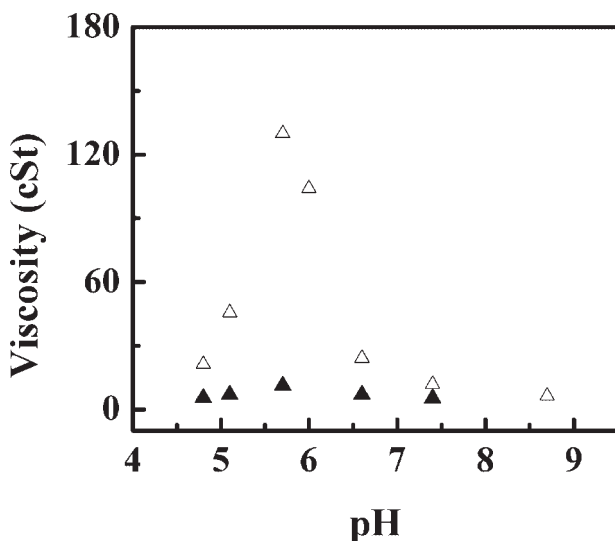


**Figure 5.** The effects of pH on the kinematic viscosity of MAb1 solution containing 130 mg/mL protein, 2–17.5 mM of acetate or arginine with (▲) and without (△) 150 mM NaCl. The viscosity was measured using a Cannon-Fenske Routine viscometer (Cannon Instrument) at 25°C.

aggregates. The size exclusion chromatography and sedimentation velocity were conducted in phosphate buffer at pH 6.8. MAb1 solutions prepared with either a high viscosity or low viscosity showed no significant difference in percentage of non-dissociable soluble aggregates (<1% total) (Table 1). In addition, the soluble aggregates formed under both conditions have a very similar size distribution as demonstrated by similar retention time and molecular mass as determined by on-line multi angle light scattering (data not shown). These results strongly suggest that the non-dissociable soluble aggregates are not responsible for the high viscosity of MAb1 since the percentage and distribution of non-dissociable soluble aggregates do not correlate with changes of sample viscosity.

Additional evidence that non-dissociable soluble aggregates have little impact on MAb1 viscosity was obtained by assessing size distribution of a MAb1 sample that had a large percentage of non-dissociable soluble aggregate. This sample was prepared by storing the lyophilized monoclonal antibody at an elevated temperature of 60°C for 1 month. Size exclusion chromatography showed that even with more than 11% of protein as soluble aggregate, the viscosity actually decreased significantly (Table 1). Although it is not well understood what caused the decrease in viscosity these results clearly demonstrate that the non-dissociable soluble aggregates do not increase viscosity of the MAb1 formulation.

#### Reversible Protein Self-Association and Protein Viscosity

The viscosity of a macromolecule solution is dependent on the concentration, shape, molecular weight and molecular interaction of the solute. The viscosity of a protein solution, can be related to the solvent viscosity,  $\eta_0$ , and concentration of the protein,  $c_p$ , in g/mL by a power series:<sup>26</sup>

$$\eta = \eta_0(1 + k_1c_p + k_2c_p^2 + \dots) \quad (3)$$

where  $k_1$  is related to the contribution from individual protein molecules,  $k_2$  and higher order coefficients are related to effects from interactions of 2, 3, or more protein molecules. The constant,  $k_1$ , is also known as the intrinsic viscosity  $[\eta]$ , and it can be obtained experimentally by extrapolating to zero protein concentration as

$$[\eta] = \lim_{c_p \rightarrow 0} (\eta_{sp}/c_p) \quad (4)$$

**Table 1.** Summary of Non-Dissociable Soluble Aggregates of MAb1 in Different Formulations

|                                                                              | Soluble<br>Aggregates<br>by SEC | Soluble<br>Aggregates<br>by AUC | Viscosity<br>(mPaS) |
|------------------------------------------------------------------------------|---------------------------------|---------------------------------|---------------------|
| MAb1 reconstituted with SWFI <sup>a</sup>                                    | 0.2%                            | 0.5%                            | 60                  |
| MAb1 reconstituted with saline <sup>b</sup>                                  | 0.3%                            | 0.6%                            | 12                  |
| Lyophilized MAb1 stored at 60°C, and<br>reconstituted with SWFI <sup>c</sup> | 11%                             | 11%                             | 41                  |

<sup>a</sup>Lyophilized MAb1 was reconstituted with 1.4 mL SWFI to final concentration of 125 mg/mL protein, 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20, at pH 6.0.

<sup>b</sup>Lyophilized MAb1 was reconstituted with 0.9% saline to final concentration of 125 mg/mL protein, 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20, 128 mM NaCl, at pH 6.0.

<sup>c</sup>Lyophilized MAb1 was stored at 60°C for 1 week and then reconstituted with 1.4 mL SWFI to final concentration of 125 mg/mL protein, 16 mM histidine, 266 mM sucrose, 0.03% polysorbate 20, at pH 6.0.

where  $\eta_{sp} = (\eta - \eta_0)/\eta_0$ . The intrinsic viscosity is dependent on shape of the protein and several methodologies have been proposed to obtain information about the conformation of macromolecules through determination of intrinsic viscosity. However, at the higher concentrations of interest, the higher order terms of the power series in Equation 3 will dominate, and the virial coefficients, which are related to molecular interactions, will significantly contribute to the viscosity.

In order to determine if reversible self-association could account for the high concentration dependency of viscosity of MAb1, this antibody was compared directly with two other IgG1 monoclonal antibodies, MAb2 and 3. These three monoclonal antibodies share essential the same constant regions of amino acid sequences, and differ only in the complementarity determining regions (CDRs). All three antibodies were formulated in the same lyophilized formulation and freeze-dried using the same lyophilization cycle. After reconstitution with water to a final MAb concentration of 85 mg/mL, 34 mM histidine, 204 mM trehalose, 0.034% polysorbate 20, the resulting viscosities were 7.6, 2.4, and 2.4 mPaS for MAb1, 2, and 3, respectively. The value of 7.6 obtained for MAb1 at 85 mg/mL under these conditions is slightly lower than values shown in Figure 1 because of the higher histidine concentration (34 mM vs. 16 mM) at pH 6, which contributes to a higher ionic strength.

Analysis by SEC did not reveal any significant differences in mass distribution between any of the MAb preparations (>99% monomer). One of the limitations in SEC analysis is the low loading concentrations often used and the dilution that occurs during the chromatography. The latter

makes it difficult to detect weakly self-associated species that can be dissociated to monomer upon dilution. To overcome this limitation sedimentation equilibrium ultracentrifugation analysis using a preparative ultracentrifuge and a micro-fractionator as described by Minton et al.<sup>27–29</sup> was used to investigate protein self-association at 100 mg/mL loading concentration and low ionic strength conditions. The resulting concentration gradient curves as a function of centrifugal radial position clearly demonstrate that of the three monoclonal antibodies, MAb1 with the highest viscosity has the steepest concentration gradient (Figure 6A). The apparent weight average molecular weight as a function of protein concentration for each monoclonal antibody was obtained from the slope of the natural logarithm of protein concentration, vs. radial position ( $\ln C$  vs.  $r^2/2$  plot, Figure 6B). Due to thermodynamic nonideality,<sup>13,24,27</sup> the apparent weight average molecular weights of antibodies are less than the monomer molecular weight of typical monoclonal antibodies at high protein concentration. The main contributors to non-ideality are excluded volume effects derived from the large size of the macromolecular solutes, and the primary charge effect due to the unsuppressed charge on the macromolecule surface. Since all three antibodies contain positive charges at pH 6.0, the steep decrease in apparent molecular weight as function of protein concentration is likely due to both the excluded volume and primary charge effects.

Analysis of the gradients to obtain corrected weight average molecular weights is complicated because of the high concentration of the protein, which results in a thermodynamically non-ideal

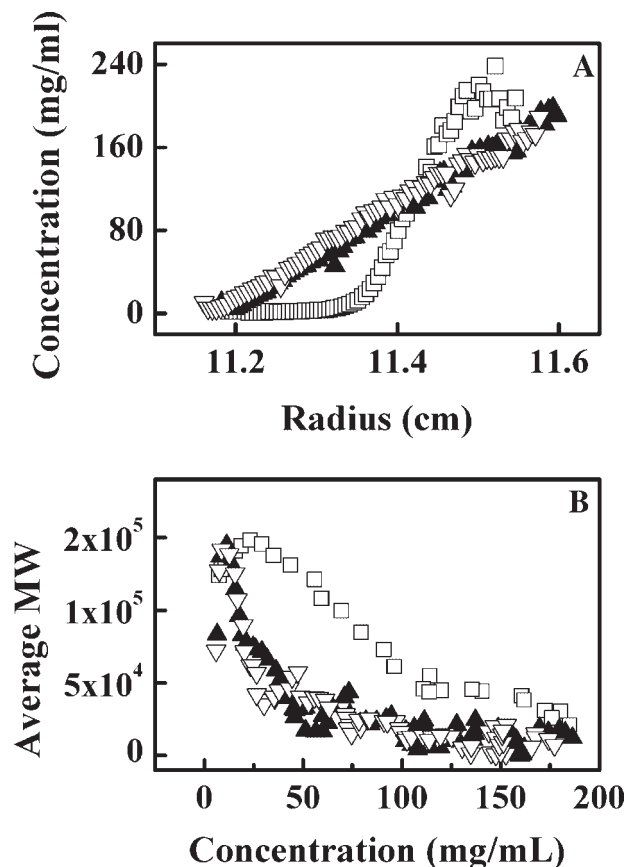


**Figure 6.** The sedimentation equilibrium data of MAb1 ( $\square$ ), MAb2 ( $\blacktriangle$ ), and MAb3 ( $\nabla$ ) at 100 mg/mL, containing 240 mM trehalose, 40 mM histidine, 0.04% polysorbate 20 (A). The sedimentation equilibrium experiments were conducted at 15000 rpm and 25°C. The apparent weight average molecular weights of MAb1 were plotted as a function of protein concentration (B).

solution. Non-ideality can be related to the second virial coefficient,  $B$  that can be resolved by evaluation of the concentration dependency of the apparent molecular weight. The second virial coefficient,  $B$  is a thermodynamic parameter that can be used to estimate protein–protein interac-

tions at high protein concentrations. A positive  $B$  value usually is indicative of either strong attractive interaction between the solute and solvent or by repulsive interactions between solute molecules (Kensal et al., 1998). A negative  $B$  value indicates protein–protein interactions are favored over protein–solvent interactions, and a very negative value of  $B$  can lead to phase separation. The use of  $B$  from sedimentation equilibrium for determination of protein–protein interaction can be complicated due to the primary charge, excluded volume effects and repulsive interaction which can result in a positive  $B$  value, while attractive interaction can lead to a negative  $B$  value. Thus, at high protein concentrations the sign and magnitude of the  $B$  value will be dependent on the relative contributions from these effects, which may coexist and offset with each other. To determine the value of  $B$ , the apparent weight average molecular weights were first calculated by a sliding linear regression whereby local slopes were computed through small regions on the  $\ln(c)$  versus  $r^2/2$  plot. The  $B$  values for the three monoclonal antibodies with or without 150 mM NaCl were then determined from a linear least squares regression of Equation 2 by using the apparent weight average molecular weights and concentrations. Analyses of MAb 2 and 3 yield positive and similar  $B$  values under the same conditions, indicating that there is no significant protein–protein attractive interaction. Furthermore the resulting values for molecular weight suggests that these monoclonal antibodies are primarily monomeric or may contain small amounts of reversible soluble aggregates in solution (Table 2). However, due to the large errors in the molecular weight determination by sedimentation equilibrium under non-ideal conditions, it is difficult to conclude that there is not any self-association of MAb2 and MAb3 under low ionic strength conditions. The lower  $B$  value for MAb2 and 3 in the presence of 150 mM NaCl reflects the fact that ionic screening effectively reduced the charge effect or

**Table 2.** Second Virial Coefficient of Antibodies with or without NaCl

| Antibody | 150 mM NaCl | Mw, Monomer (kDa) | $B$ (cm <sup>3</sup> /g <sup>2</sup> -mole) $\times 10^4$ | Linear Correlation Coefficient, $R$ |
|----------|-------------|-------------------|-----------------------------------------------------------|-------------------------------------|
| MAb1     | Yes         | 130 (101–166)     | $2.3 \pm 0.12$                                            | 0.88                                |
| MAb2     | No          | 167 (132–234)     | $2.2 \pm 0.10$                                            | 0.96                                |
| MAb3     | No          | 200 (164–274)     | $2.2 \pm 0.11$                                            | 0.97                                |
| MAb2     | Yes         | 150 (136–166)     | $0.8 \pm 0.04$                                            | 0.97                                |
| MAb3     | Yes         | 143 (133–156)     | $1.1 \pm 0.06$                                            | 0.98                                |

any repulsive charge interactions between protein molecules, and the resulting molecular weights are consistent with those of monomers. The second virial coefficient determination for MAb1 is more complicated due to the non-linear relationship between  $1/M_{w,app}$  and protein concentration under low ionic strength conditions. The data cannot be fitted with a single non-ideal species model, suggesting the presence of large, self-association species. The  $B$  value is slightly negative or near zero at low protein concentration, and becomes positive at higher concentrations. This suggests that the protein interactions, charge and excluded volume effects may coexist at the same time. At the low protein concentration range the attractive forces dominate, and therefore the value of  $B$  is negative, whereas at higher concentration the charge and excluded volume effects increase and result in a positive  $B$  value. The attractive electrostatic interaction between MAb1 molecules can be largely overcome by increasing salt concentration. As shown in Table 2, the  $B$  value for MAb1 in 150 mM NaCl is positive, indicating the lack of attractive electrostatic interactions between protein molecules.

To determine the extent of the self-association of MAb1, a semi-empirical method was developed. It was assumed that MAb2 and MAb 3 have a similar structure as MAb1, and that they are not associating to any great extent in solution. It was further assumed that the non-ideality corrections for MAb2 and 3 could be applied to the analysis of MAb1. Two lines of evidence support these assumptions. First in the presence of 150 mM NaCl the three monoclonal antibodies have the same sedimentation equilibrium concentration gradient suggesting that the correction due to excluded volume effects is the same for all three antibodies (Figure 7). The assumption that MAb2 and 3 are not undergoing any appreciable self-association is supported by analysis of the monoclonal antibody solutions using a hard quasispherical model as suggested by Ross and Minton.<sup>30</sup> The viscosity,  $\eta$  of a concentrated protein solution is related to the protein concentration,  $c$  by a semiempirical equation:

$$\eta = \eta_0 \exp\{[\eta] c / (1 - (k/v)[\eta]c)\} \quad (5)$$

where  $\eta_0$  is the solvent viscosity,  $[\eta]$  is the intrinsic viscosity of the protein,  $k$  is a "crowding factor," and  $v$  is the Simha parameter related to the ellipsoid of revolution used to model the protein.<sup>31</sup> Using a typical value of  $6.3 \text{ cm}^3/\text{g}$  for the intrinsic viscosity of an IgG<sub>1</sub>,<sup>15,32</sup> and a determined sol-

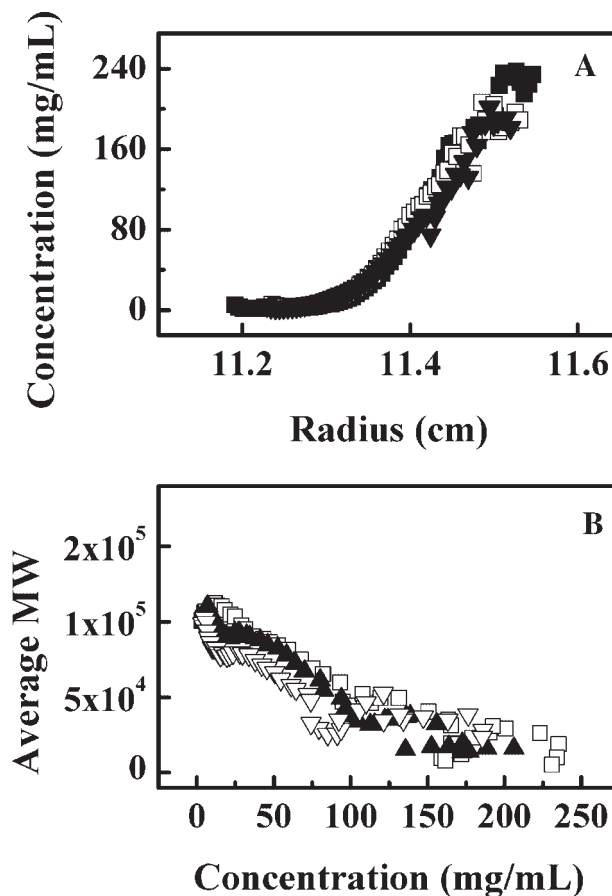


**Figure 7.** The sedimentation equilibrium data of MAb1 (■), MAb2 (□), and MAb 3 (▼) at 100 mg/mL, containing 240 mM trehalose, 40 mM histidine, 0.04% polysorbate 20, 150 mM NaCl (A). The sedimentation equilibrium experiments were conducted at 15000 rpm and 25°C. The apparent weight average molecular weights of MAb were plotted as a function of protein concentration (B).

vent viscosity of 1.1 mPas, data from the three monoclonal antibodies were analyzed using Equation 5 and non-linear regression (Kaleidagraph<sup>®</sup>, Synergy Software) with  $k/v$  as a fitting parameter (Figure 8A–C). The concentration dependency of MAb 2 and 3 is shown in Figure 8A. Samples were exchanged and concentrated into 266 mM sucrose and 16 mM histidine at pH 6.0 with and without 150 mM NaCl. In addition MAb2 was prepared as a lyophilized sample at 25 mg/mL MAb2, 60 mM trehalose, 10 mM histidine and 0.01% polysorbate 20 at pH 6. The lyophilized samples were reconstituted with water to either 100 mg/mL MAb2 in 240 mM trehalose, 40 mM histidine 0.04% polysorbate 20 or 125 mg/mL in 300 mM trehalose, 50 mM histidine, and 0.05% polysorbate 20. The

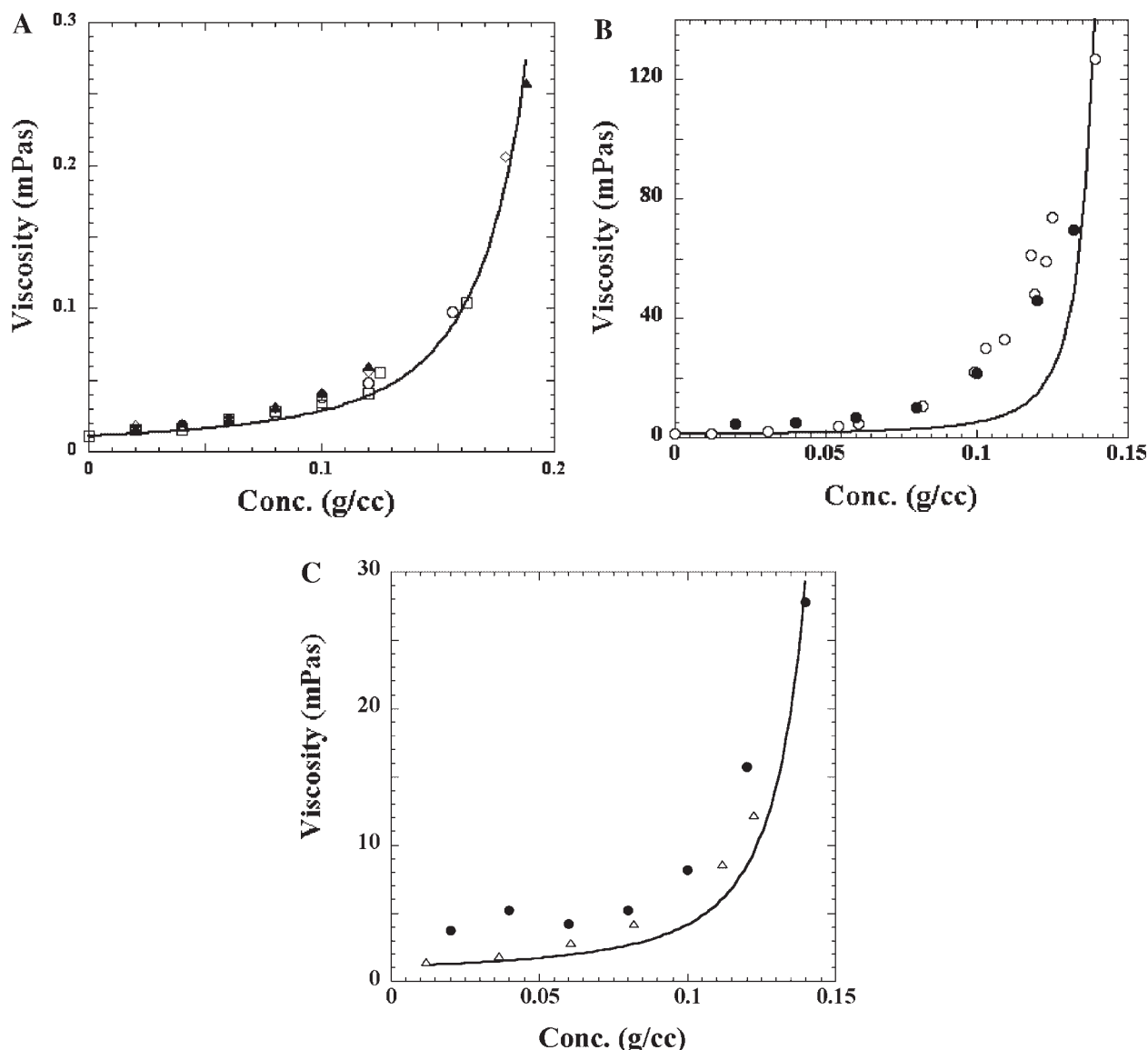


**Figure 8.** Viscosity of MAb as a function of protein concentration. (A): MAb2 with no added salt (□) and at 150 mM NaCl (○). MAb3 with no added salt (▲), and at 150 mM NaCl (◇). Non-lyophilized samples prepared by TFF have a composition of 266 mM sucrose and 16 mM histidine at pH 6.0 with and without 150 mM NaCl. Lyophilized and reconstituted MAb2 with no added salt (■). Lyophilized samples are at either 100 mg/mL MAb2 in 240 mM trehalose, 40 mM histidine, 0.04% polysorbate 20 or 125 mg/mL MAb2 in 300 mM trehalose, 50 mM histidine, and 0.05% polysorbate 20. The solid curve is the result of a non-linear regression of all the data to Equation 5 using a solvent viscosity of 1.1 mPas and intrinsic viscosity of 6.3 cm<sup>3</sup>/g. The best fit with a  $X^2 = 0.003$  resulted in a value for  $k/v = 0.533$ . (B): MAb1 lyophilized and reconstituted (○) and non-lyophilized (●) without any added NaCl. All samples contain 266 mM sucrose, 16 mM histidine, and 0.03% polysorbate 20 at pH 6. The solid curve is the result of a non-linear regression of all the data to Equation 5 using a solvent viscosity of 1.1 mPas and intrinsic viscosity of 6.3 cm<sup>3</sup>/g. The best fit with a  $X^2 = 1.16$  resulted in a value for  $k/v = 0.936$ . (C): MAb1 lyophilized and reconstituted (△) and non-lyophilized (●) at 150 mM NaCl. All samples contain 266 mM sucrose, 16 mM histidine, and 0.03% polysorbate 20 and 150 mM NaCl at pH 6. The solid curve is the result of a non-linear regression of all the data to Equation 5 using a solvent viscosity of 1.1 mPas and intrinsic viscosity of 6.3 cm<sup>3</sup>/g. The best fit with a  $X^2 = 0.012$  resulted in a value for  $k/v = 0.829$ .

resulting viscosity as shown in Figure 8A is neither dependent on the choice of sugar or presence of polysorbate 20 (at least in the range investigated) nor whether the samples were lyophilized and reconstituted.

The concentration dependency of MAb2 and MAb3 is nicely accounted for using the hard quasispherical model, which assumes no protein–protein attractive interactions (Figure 8A). Moreover, the addition of NaCl has little impact on the viscosities of either MAb2 or MAb3 strongly suggesting that repulsive electrostatic interactions do not play a major role in the increase of viscosity as a function of protein concentration, which is consistent with the results of the ionic strength dependency of the viscosity discussed earlier. Therefore, non-ideality is mainly due to excluded volume effects, which accounts for the viscosity increase as a function of concentration.

The viscosity dependence of MAb1 with and without added salt is shown in Figure 8B and C. Lyophilized samples were reconstituted and diluted with buffered solutions with and without NaCl to yield the same composition as the non-lyophilized preparations (266 mM sucrose, 16 mM histidine, and 0.03% polysorbate 20). The concentration dependency of the viscosity of MAb1 cannot be fitted with the hard quasispherical model demonstrating that protein–protein interactions contribute to the unusual high viscosity observed for this monoclonal antibody (Figure 8B). Addition of salt resulting in an increase in ionic strength decreases the viscosity of MAb1, but not to the same levels for MAb 2 and MAb3 (Figure 8C). Although the fit to the hard quasispherical model is better than in the absence of added salt, the fit is not as good compared to the fit for MAb2 and MAb3. This suggests that addition of NaCl to a final concentration of 150 mM does not result in complete disruption of the attractive electrostatic interactions in MAb1 preparations responsible for the high viscosities observed. In all of these studies there was very little difference between lyophilized and non-lyophilized samples again demonstrating that the lyophilization process does not impact the determined viscosities of the preparations.

Assuming that MAb2 and 3 exist as monomeric molecules in solution, the non-ideality correction for the charge and excluded volume effects can be obtained in the absence of added NaCl for an IgG1 monomer with 150 kDa molecular weight. These corrections are obtained from the relationship<sup>11</sup> between apparent molecular weight,  $M_a$  at weight/

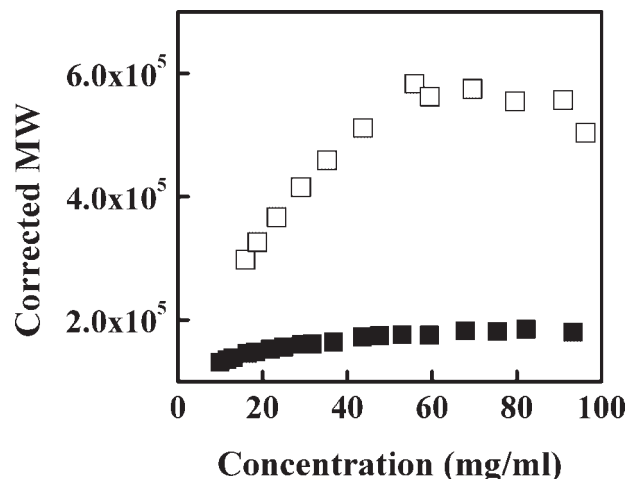


**Figure 9.** The corrected average molecular weight of MAb1 at 100 mg/mL, containing 240 mM trehalose, 40 mM histidine, 0.04% polysorbate 20 with no added salt (□) and with 150 mM NaCl (■) as function of protein concentration.

volume concentration  $c$  and actual molecular weight,  $M$ :

$$M_a = M[1 + c(\ln\gamma/dc)] \quad (6)$$

where  $\gamma$  is the activity coefficient of the monoclonal antibody. Assuming that MAb2 and 3 are essentially monomeric in solution leads to a multiplicative correction factor as a function of  $c$  when  $M$  is set equal to 150 kDa.

Applying these corrections to MAb1 then results in the corrected molecular weight as a function of concentration (Figure 9). These corrections are estimates and probably do not account for the complete correction, yet they clearly demonstrate that MAb1 undergoes self-association as a function of concentration. After adjustment of the formulation for MAb1 to 150 mM NaCl these interactions are greatly reduced (Figure 9). The reduction of these reversible protein–protein interactions results in a reduction of viscosity (Figure 3), and in fact, demonstrates that reversible self-association can have a major impact on the physical properties of a protein formulation, and that protein charge interactions may play a crucial role in this behavior.

## CONCLUSIONS

Analysis at high protein concentrations requires methods that do not result in dissociation of non-covalent low affinity complexes due to dilution of

the formulation. The reversible self-association of a particular monoclonal antibody appears to be mediated mainly by electrostatic interactions of charged residues or dipole moments on the surface of the protein. These multi-valent low affinity interactions can result in unusually high viscosity of the concentrated monoclonal antibody in low ionic strength buffers. Despite reversibility, such protein self-association can have a major impact on important pharmaceutical properties.

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