

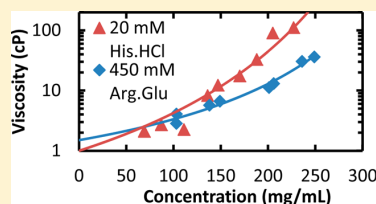
Viscosity Reduction of a Concentrated Monoclonal Antibody with Arginine·HCl and Arginine·Glutamate

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Supporting Information

ABSTRACT: To further advance a subcutaneous injection of monoclonal antibodies (mAbs) at elevated concentrations, novel concepts are needed to lower the viscosity. The addition of high concentrations of cosolutes, namely, arginine glutamate (Arg-Glu) or Arg-HCl, reduced the viscosity of a ~250 mg/mL mAb solution up to 6-fold. With Arg-Glu, the viscosity of the mAb solution was reduced to 30 cP and for a polyclonal sheep IgG solution to 17 cP both at ~250 mg/mL. Viscosities went through a maximum at the mAb isoelectric point for solutions with Arg-Glu or Arg-HCl. In contrast the viscosity was only weakly affected by NaCl or the preferentially excluded molecule trehalose. The large viscosity reduction from Arg may be attributed to direct binding to the mAb, resulting in suppression of both hydrophobic and local anisotropic electrostatic attraction. Aggregate formation was negligible for high cosolute mAb solutions as demonstrated by SEC even after 8 weeks of 25 °C storage.



INTRODUCTION

Monoclonal antibodies (mAbs) are of great interest for the treatment of a wide range of diseases including various types of cancer, Alzheimer's disease, asthma, and numerous autoimmune diseases. Given that intravenous administration of dilute mAb solutions is time-consuming and requires medical supervision, patients would prefer either oral administration, which is currently an area of active research,¹ or subcutaneous (SC) self-injection.^{2,3} Since the volume is limited to ~1–2 mL in SC administration, the recommended dosage often requires concentrations greater than 150 mg/mL mAb where viscosities can exceed the desired level of 20 cP.^{2,4,5} For example, the viscosities at high concentration have been shown to increase markedly for a series of mAbs as the interactions (measured at low concentration by dynamic or static light scattering) change from repulsive to attractive, for a given buffer/cosolute system.^{6–10} The elevated viscosities often arise as a result of interactions between Fab (antibody binding fragment) regions or between Fab and Fc (crystallizable fragment) regions, often involving the CDRs (complementarity determining regions).^{4,6,11–15}

Often mAbs with isoelectric points (pI) between pH 7 and 9 are formulated in a buffer solution at pH 5–6, for example in 20–30 mM histidine (His),^{2,6–9,15–17} such that the net charge provides electrostatic repulsion to favor stability against aggregation. At high concentrations (150 to 300 mg/mL), the distance between the surfaces of neighboring antibodies is on the order of the protein size (~1–5 nm). Here, local anisotropic attractive interactions between multiple charges and dipoles as well as hydrophobic interactions may produce aggregates or protein networks that raise the viscosity. For example, the formation of protein oligomers may produce a

large increase in the viscosity given that they occupy a greater volume than monomeric protein as a function of the fractal dimension.^{16,18–21} Here, the highly viscous networks at low shear rates may be partially disrupted at elevated shear rates, resulting in shear thinning behavior.^{15,22–24} Even for irreversible aggregates composed of a few proteins, shear thinning behavior may be present as seen for bovine serum albumin (BSA) and for mAbs at low concentrations.^{25–27} Although the viscosity of mAb formulations may be lowered by engineering the amino acid sequence to weaken the aforementioned attractive interactions,^{7,22} this approach is time-consuming, highly specific to each particular mAb and can influence the mAb therapeutic efficacy.^{7,22} In some cases, salts may be used to screen attractive anisotropic electrostatic interactions and thus lower the viscosity, particularly chaotropic salts.^{6,15,16,28–30}

Recently, there has been significant interest in utilizing organic cosolutes to modify protein–protein interactions (PPI) to attempt to lower the viscosity.^{20,31–34} High concentrations of a neutral cosolute, trehalose, were used to tune the depletion attraction between proteins to favor the folded state and enhance protein stability as described by a free energy model,^{31,32,35} and coarse grained simulations.^{36,37} For several proteins including mAbs where inorganic salts had little effect, organic salts containing large hydrophobic regions lowered the viscosity several fold at concentrations of 0.25 to 0.5 M, by weakening both the electrostatic and hydrophobic interac-

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tions.^{28,34} Finally, low viscosities have been observed for insoluble micrometer-sized particulates of mAbs suspended in organic solvents^{3,5} or aqueous buffers containing a high organic solvent fraction.³⁸

Arginine (Arg), particularly in the protonated state, is well-known to reduce protein self-association^{20,21,39–41} and provide stability against irreversible aggregation.^{39,42–44} At high concentrations, Arg binds directly to protein surfaces, as shown by static light scattering.²¹ Relative to Arg-HCl, the stability of mAbs may be improved using a mixture of arginine and glutamic acid, whereby a proton is transferred from Glu to Arg.^{43,44} These ions interact with the charged surface residues on the proteins increasing the concentrations of both ions at the protein surface.⁴⁵ The weakening of attraction between protein molecules likely contributes to observed reductions in viscosity upon the addition of 150 mM,⁴⁶ 200 mM,⁷ and 500 mM³⁴ Arg-HCl to 150–200 mg/mL mAb solutions. In a rare example of a study that examined a mAb concentration above 200 mg/mL, in this case 250 mg/mL, the addition of 300 mM Arg-HCl was shown to reduce the viscosity of the mAb solution from 80 to 40 cP.⁴⁷ In each of these studies only a single Arg concentration was studied. In rare instances, the viscosity has been investigated as a function of protein concentration, Arg concentration, and pH, for example, for polyclonal human and bovine gamma globulin (HGG and BGG) at concentrations up to ~300 mg/mL.³³ However, for these polyclonal antibodies the viscosities were relatively low even without added cosolute. Furthermore, the pH effect is complicated given the polydispersity in isoelectric points for the component gamma globulins. Despite significant study, it is still unclear why Arg destabilizes some mAbs⁴³ and increases the viscosity of others.⁴ It remains to be determined how the effects of Arg on viscosity and stability are related, and if Arg can produce large improvements in both the viscosity and stability for the same mAb as a function of pH and the presence of other excipients, such as lyoprotectants, for example trehalose. Additionally, the effects of Arg-Glu (salt formed from arginine and glutamic acid, not to be confused with the dipeptide) on protein viscosity have received little attention even though Arg-Glu has been shown to consistently improve stability relative to Arg-HCl.^{43–45}

Herein, we examine the effect of the cosolutes NaCl, trehalose, Arg-Glu, and Arg-HCl on the viscosity and stability of highly concentrated mAb solutions (~250 mg/mL) at pH values ranging from 5 to 11 (pI = 9.3). The concentrated solutions were formed by centrifugal filtration with 30 and 50 kDa cutoff filters after buffer exchange. The pH was varied from below to above the isoelectric point to modify the protein charge distribution and thus the PPI and the protein-cosolute interactions, in order to gain insight into the viscosity behavior. The addition of depletion attraction with trehalose was not found to lower the viscosity and the reduction was modest by screening with added NaCl. In contrast, the viscosity of a solution of mAb1 was reduced markedly in the presence of 450 mM Arg-Glu or Arg-HCl, indicating the importance of direct cosolute binding;²¹ whereby, the bound cosolute may weaken local anisotropic electrostatic attraction and hydrophobic interactions. The shear rate was varied from 0.08 to 800 s⁻¹ for the Arg-Glu system and compared with a low cosolute control to further examine the effects of Arg-Glu on protein networks. Finally, the mAb1 formulations are shown to be stable against loss of monomer after storage for up to 8 weeks according to size exclusion chromatography (SEC).

MATERIALS AND METHODS

Materials. The monoclonal antibody used in this study (mAb1) was an IgG1 obtained from AbbVie at 120 mg/mL in a proprietary buffer composition. Arginine, glutamic acid, lysine, acetic acid, sodium glutamate, arginine hydrochloride, proline, glycine, histidine, histidine hydrochloride monohydrate, sodium phosphate monobasic monohydrate, sodium phosphate dibasic, sodium bicarbonate, and HCl were purchased from Fisher Scientific, Fairlawn, NJ. Trehalose was purchased from Ferro Pfanstiehl Laboratories Inc., Waukegan, IL. Amicon Ultra-15 Ultracel-30K and Amicon Ultra 0.5 Ultracel-50K centrifugal filters were purchased from Merck Millipore Ltd. Ireland.

Buffer Exchange. Initially, 0.4 mL of the 120 mg/mL mAb1 solution was diluted to 4 mg/mL in a buffer containing the desired concentrations of cosolutes (12 mL total, initial buffer volumetric fraction is 3.33% (0.4 mL out of 12)). The resulting solution was then filtered using a Millipore Centricon centrifugal concentrator tube with a molecular weight cutoff of 30 kDa and a capacity of 12 mL at a spin speed of 4500 rcf for 12 min. The protein solution was concentrated until the solution volume dropped to about 5 mL with a protein concentration of ~10 mg/mL. Then the retained protein solution was again diluted using the desired solution buffer to make up the volume to 12 mL (initial buffer volume fraction reduced to 1.4% (3.33% out of 5 mL in 12 mL)) and then centrifuged again. This process was repeated 4 or more times until the volume of flow through was about 40 mL and the volumetric fraction of initial buffer was less than 1% assuming ideal mixing. After this, the solution was further concentrated by continuing the centrifugation so that the final volume was about 0.5 mL at about 80 mg/mL. Alternatively in Table 1,

Table 1. Viscosity of mAb1 Solutions at 130–150 mg/mL

protein conc (mg/mL)	cosolutes	cosolute conc (mM)	pH	η_0 (cP)	η (cP)	η_{inh} (mL/g)
146			6.5	1.0	20 ± 1.5	20.7
130	Tre	150	6.5	1.0	15 ± 0.1	20.9
132	Pro	150	6.5	1.0	13 ± 0.5	19.3
139	NaCl	150	6.5	1.0	7.8 ± 0.2	14.8
139	Lys-HCl	150	6.5	1.0	9.5 ± 0.1	16.2
134	Na-Glu	150	6.5	1.0	8.5 ± 0.5	16.0
134	Arg-HCl	150	6.5	1.0	6.4 ± 0.3	13.8
133	Arg-HCl	75	6.5	1.0	6.9 ± 1.0	14.6

buffer exchange was carried out in DI water and the cosolutes were added after centrifugal concentration to 155 mg/mL by mixing concentrated aqueous solutions of cosolute into the protein solution.

Centrifugal Concentration to >200 mg/mL. Tare weights were taken of the individual components (filter, permeate tube, and retentate tube) of the centrifugal filter assembly (Millipore Microcon, Ultracel YM-50 membrane, 50 kDa nominal molecular weight limit, 0.5 mL capacity). After buffer exchange, the desired volume of 80 mg/mL protein solution was pipetted into the retentate chamber. The filter assembly was then centrifuged (Eppendorf Centrifuge 5415D) at either 5000 or 10000 rcf, typically for about 40 min in 5–10 min increments with volume monitoring at every stop until the calculated final volume for the desired final concentration was reached. The volume measurements were done by weighing both the retentate and permeate. The protein solution in the

retentate was recovered by inverting the filter assembly into a retentate recovery tube, and centrifuging for 2 min at 1000 rcf. The resulting solution was transferred to a 0.1 mL conical vial (V-Vial, Wheaton), and the concentration was verified spectrophotometrically by withdrawing a small amount (2 μ L) of the sample (described in more detail below). The concentration of cosolutes may change during filtration;⁴⁸ however, the changes become relatively small for the high cosolute solutions due to ionic screening (explained in the [Supporting Information](#)). Further evidence for the small change was a minimal change observed in the pH by less than 0.02 units throughout filtration. In contrast, the pH of an example low cosolute sample drifted by $\sim$ 0.2 units from 5.48 at the start to 5.67 at the end of the run.

In cases where a larger sample was needed to perform sterile filtration on the solutions, the entire centrifugal filtration run was conducted in larger centrifuge tubes. A larger amount of protein (170 mg) was initially loaded onto the filter at the start of buffer exchange. Instead of transferring the sample to the smaller 0.5 mL centrifuge filters, it was transferred to another Millipore Centricon centrifuge concentrator tube and then concentrated to the desired final volume at 4500 rcf. The sample was characterized in the same way as the smaller samples, and then sterile filtered using a 1 mL syringe (Becton Dickinson & Co. with Luer-Lok tip) and a syringe filter with a 0.22 μ m cutoff. The sample was also weighed before and after filtration to determine the yield for the sterile filtration step.

Characterization of the Protein Solutions. Viscosity Measurement. The viscosities were measured, after temperature normalization to 25 $^{\circ}$ C, in triplicate using a 25 gauge (ID = 0.1 mm) 1.5 in. long needle (Becton Dickinson & Co. Precision Glide Needle) attached to a 1 mL syringe (Becton Dickinson & Co. 1 mL syringe with Luer-Lok tip), on the basis of the Hagen–Poiseuille equation. The reported viscosities are the average of the three measurements along with the standard deviation. The flow rate of sample through the needle was determined using the estimated volume which was correlated to the height of the liquid in the conical vial ([Figure S1](#)) and measuring the time taken for the meniscus height to move between two points. This flow rate was correlated to viscosity from a calibration curve derived from a set of standards of known viscosities as shown in [Figure S2](#). Cone and plate rheometry experiments were conducted on a standard torsional rheometer (AR2000EX, TA Instruments) with a 40 mm diameter cone with 2 $^{\circ}$ of angle and a truncation gap of 55 μ m. The cone-and-plate geometry is selected for the constant shear rate in the tool-plate gap. Sample temperature was controlled by a Peltier plate set to 25 $^{\circ}$ C.

Dynamic Light Scattering (DLS) for Stability and Diffusion Interaction Parameters. The hydrodynamic diameter of mAb1 in solution was measured by dynamic light scattering (DLS) after dilution to 0.5 mg/mL to check for irreversible aggregates. The reported hydrodynamic diameters are the average volume-based particle size distributions to the DLS data from the algorithm CONTIN. The DLS was run at an angle of 90 $^{\circ}$ with a Brookhaven ZetaPlus. The samples were pipetted into a Uvette (Eppendorf) which was then mounted in the instrument to conduct three replicate runs of 2 min each. The diffusion interaction parameter (k_D) was obtained by fitting the diffusion coefficients at 2, 5, 10, 15, and 20 mg/mL with the equation $D = D_0(1 + k_D c)$ where D is the measured diffusion coefficient, D_0 is the diffusion coefficient at infinite dilution, and c is the protein concentration.

Size Exclusion Chromatography (SEC). For analysis of noncovalent aggregates, the sample was diluted to 1 mg/mL in the mobile phase (100 mM sodium phosphate, 300 mM sodium chloride, pH 7). A volume of the diluted sample containing 20 μ g of mAb1 was analyzed with a Waters Breeze HPLC, using TOSOH Biosciences TSKgel3000SW_{XL} and TSKgel2000SW columns in series, with eluate monitored by absorbance at 214 nm.

Protein Concentration Determination. For determining the protein concentration of a given solution, 2 μ L of sample was measured out and diluted into a receiving vessel containing 998 μ L of 50 mM pH 6.4 phosphate buffer and mixed well with the pipet tip and gentle tilting. The diluted samples were prepared in duplicate, and the average deviation between replicate samples was <5% of the measured protein concentration. The absorbance of the resulting solution was measured using a Cary 3E UV–visible spectrophotometer in a cuvette (Hellma cells) with a path length of 1 cm. Then using Beer's law ($A = \epsilon bc$, where ϵ = extinction coefficient = 1.42 mL mg $^{-1}$ cm $^{-1}$ at 280 nm, b = path length = 1 cm), knowing the absorbance, the concentration of protein in the solution was calculated.

Sample Storage. The storage stability was examined in the 0.1 mL conical vials, used for viscosity measurements, sealed with the vial cap and parafilm in a -40 $^{\circ}$ C freezer, in a refrigerator at 4 $^{\circ}$ C and on the bench at room temperature. The sample for each time-point was taken from a separate vial which was then discarded.

RESULTS

Effect of Cosolutes on Viscosity at $\sim$ 150 mg/mL mAb Concentration. To provide a basis for understanding the viscosity experiments at $\sim$ 250 mg/mL protein, we first conducted experiments at a lower protein concentration of $\sim$ 150 mg/mL. The effects of various cosolutes on viscosity (η) were tested by adding them at 150 mM to a 150 mg/mL solution of mAb1 in DI water, as shown in [Table 1](#). Note that the addition of the cosolutes reduced the mAb1 concentration to $\sim$ 135 mg/mL.

To compare protein solutions with different concentrations we will often use the inherent viscosity $\eta_{inh} = \frac{\ln(\eta / \eta_0)}{c}$ where η_0 is the solvent viscosity, because it may be shown to change more gradually with concentration than η .⁴⁹ The Ross–Minton equation

$$\frac{\eta}{\eta_0} = \exp\left(\frac{[\eta]c}{1 - [\eta]_{\nu}^k c}\right) \quad (1)$$

where $[\eta]$ is the intrinsic viscosity (in mL/mg when concentration, c , is in mg/mL), k is the crowding factor, and ν is the Simha parameter (shape determining factor) is commonly used to model the viscosity of mAb solutions.^{6,18,50,51} Substitution into the definition of η_{inh} yields

$$\eta_{inh} = \frac{[\eta]}{1 - [\eta]_{\nu}^k c} \quad (2)$$

which is only weakly dependent upon c . The η_{inh} values at approximately 135 mg/mL mAb1 are shown in [Table 1](#) along with c and η . Upon formulation in DI water in the absence of any cosolutes, mAb1 had a η of 20 cP at 146 mg/mL resulting in an η_{inh} of 20.7 mL/g. The addition of the uncharged

cosolutes trehalose (Tre), a disaccharide, and proline (Pro), an amino acid, at 150 mM only weakly shifted η_{inh} to 20.9 and 19.3 mL/g, respectively. Alternatively, the ionic cosolutes, NaCl, Lys-HCl, Na-Glu, and Arg-HCl, all caused reductions in η_{inh} , with the largest reductions for Arg-HCl, which reduced the η down to 6 cP with a η_{inh} of 13.8 mL/g. Reducing the Arg-HCl concentration in half to 75 mM increased the η relative to 150 mM Arg-HCl, but it was still lower than for any other cosolute at 150 mM. Since the lowest viscosities were achieved with Arg, it was used as the main cosolute for the experiments at high (~ 250 mg/mL) mAb concentrations.

Effect of Cosolutes on Viscosity at ~ 250 mg/mL mAb Concentration at pH ~ 5.5 . Experiments were conducted over a wide range of mAb1 concentrations as shown in a semilog plot in Figure 1A, for a 20 mM low cosolute His-HCl

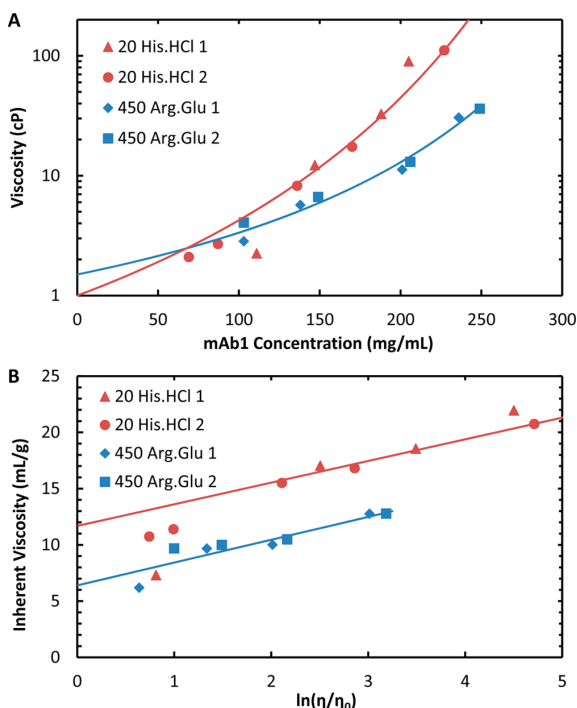


Figure 1. (A) Viscosity of mAb1 solutions as a function of the protein concentration with fits to the Ross–Minton equation. (B) Linearized Ross–Minton equation fits with same data from panel A. The Arg-Glu samples contain 450 mM Arg and 485 mM Glu while the histidine buffer samples contain 20 mM His-HCl, both samples are at pH 5.5.

Table 2. Viscosity for mAb1 Solutions with Low Cosolute

protein concn (mg/mL)	buffer components	buffer concn (mM)	pH	η_0 (cP)	η (cP)	η_{inh} (mL/g)
197 $\pm$ 15	none		6.5	1.0	56 $\pm$ 3.7	20.4
228 $\pm$ 16	His-HCl	20.0	5.5	1.0	110 $\pm$ 14	20.7
238 $\pm$ 7.2	His-HCl	30.0	6.0	1.0	190 $\pm$ 7.8	22.0
229 $\pm$ 2.8	His-HCl	30.0 (+150 mM NaCl)	6.0	1.0	61 $\pm$ 3.1	18.0
205 $\pm$ 9.6	His-HCl	50.0	6.0	1.0	56.5 $\pm$ 2.4	19.7
191 $\pm$ 1.0	phosphate	50.0	6.5	1.0	61 $\pm$ 3.8	21.5
206 $\pm$ 1.0	phosphate	50.0	8.0	1.0	85 $\pm$ 11	21.6
185 $\pm$ 8.2	carbonate	50.0	11	1.0	110 $\pm$ 6.8	25.4

buffer control along with a high cosolute system containing 450 mM Arg and 485 mM Glu, both at pH 5.5. The high cosolute system has an osmolality of approximately 3 $\times$ isotonicity,^{2,52} but studies have suggested that this range may be acceptable for injection without causing increased patient pain.^{53,54} The detailed data including η_{inh} are shown in Table S1 and Table S2. The reproducibility was within 10% for the two replicates shown for each sample at higher concentrations, given the slight variation in protein concentration. The viscosities for both the low cosolute buffer and high cosolute samples were well below 10 cP at low mAb concentrations (≤ 125 mg/mL). The η of high cosolute samples was ~ 35 cP at 250 mg/mL and several times lower than the value of 150 cP at 230 mg/mL measured for the low cosolute samples. Two additional control samples with low His-HCl buffer at the similar pH values of 5.5 and 6.0 were prepared to examine the reproducibility as shown in Table 2, along with some other low cosolute controls. These samples, covering a range of 20 to 50 mM buffer and pH values from 5.5 to 11, all exhibited the same trends as observed in Figure 1 with a $\eta \approx 100$ cP at concentrations greater than 200 mg/mL, much larger than the Arg-Glu solutions. Upon addition of 150 mM NaCl to screen electrostatic interactions, the η_{inh} only decreased by about 15–20%, which is significantly less than the decrease with the addition of Arg-Glu, even at 150 mM shown in Table 3. In Figure 1, curves were fit with the Ross–Minton eq 1 and its linearized version, eq 3.

$$\eta_{\text{inh}} \equiv \frac{\ln(\eta/\eta_0)}{c} = [\eta] + [\eta] \frac{k}{\nu} \ln(\eta/\eta_0) \quad (3)$$

Table 3. Viscosity of mAb1 Solutions as a Function of Increasing Concentration of Arg-Glu^a

protein concn (mg/mL)	Arg concn (mM)	Glu concn (mM)	η_0 (cP)	η (cP)	η_{inh} (mL/g)
283 $\pm$ 15	150	170	1.2	170 $\pm$ 16	17.4
246 $\pm$ 18	225	245	1.2	49 $\pm$ 1.7	15.0
245 $\pm$ 24	300	325	1.3	37 $\pm$ 2.1	13.5
241 $\pm$ 0.2	375	405	1.4	37 $\pm$ 4.6	13.6
263 $\pm$ 13	450	485	1.5	30 $\pm$ 1.5	11.4

^aThe pH of all of the samples was 5.5. The uncertainty is $\pm$ one standard deviation over multiple measurements in both concentration and viscosity.

As shown in Figure 1B, the plot according to eq 2B is linear for Arg-Glu samples with regressed values of $[\eta]$ and k/ν of 6.4 mL/g and 0.32, respectively. In contrast, the behavior is nonlinear for the 20 mM His-HCl control samples indicating attractive interactions beyond hard quasi-spherical interactions. Thus, the addition of Arg-Glu appears to weaken the attractive

interactions consistent with the reduction in viscosity,^{15,18,55} as attractive interactions may cause mAb oligomerization.^{16,18} This ability of Arg to break up mAb oligomers in solution has been quantified by static light scattering.²¹

The effect of Arg-Glu concentration (225 to 450 mM) on η was also studied by fixing the pH at 5.5 with a fixed molar ratio of Arg to Glu as 0.9:1 with a mAb concentration of ~250 mg/mL (Table 3). The η_{inh} decreased with increasing total cosolute concentration as shown in Figure 2 with replicate data shown in

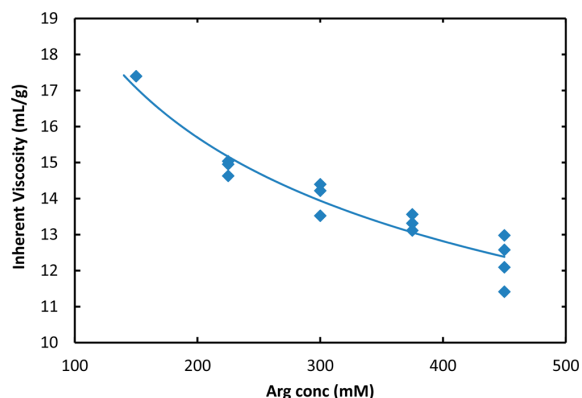


Figure 2. Decrease in inherent viscosity of mAb1 solutions as the concentration of Arg-Glu at pH 5.5 is increased. The concentration of Glu is 1.1 times the Arg concentration. The line is a guide to the eye.

Table 3 and Supporting Information Table S3. The η was as low as 30 cP at 263 mg/mL at the highest Arg-Glu concentration. Over this range of Arg concentration (150 to 450 mM) the overall η decreased by 4–5 $\times$ despite the ~25% increase in η_0 suggesting a weakening of protein–protein interactions.

Similar experiments were scaled up to ~500 μ L samples by using 15 mL filters to enable sterile filtration of the solutions, shown in Table 4. Three pairs of rows are shown where the first is before filtration and the second in bold is after sterile filtration. In each case the protein concentration dropped ~10% due to losses from adsorption to the filter while the sterile filtration yield was ~60% due to losses to filter hold-up. Although this reduction in concentration lowered η ; η_{inh} did not change significantly, suggesting that sterile filtration did not significantly alter the solutions. Therefore, to conserve protein, all additional samples were tested without sterile filtration. Additionally, the sample normalized turbidity is reported as the background corrected extinction of the fully concentrated sample at 350 nm divided by the mAb concentration and the sample path length of 0.2 cm. The turbidity spectra of these samples from 300 to 700 nm are shown in Figure S3. The

normalized turbidities were nearly twice as high for the low concentration Arg-Glu samples relative to the higher ones with more concentrated Arg-Glu. The higher turbidities are possibly a result of increased protein self-association at the lower Arg concentrations due to fewer bound Arg molecules screening attractive interactions, which is also likely the cause of the higher η s.²¹

Effect of pH and Cosolutes on Viscosity at ~250 mg/mL mAb Concentration. The effect of pH was examined for a series of mAb1 solutions containing a total concentration of 150 mg/mL Arg and Glu. The Arg charge varies from +1 between pH 5 and pH 8, to neutral between pH 8 and pH 11 and to negative above pH 11, while mAb1 had a net positive charge below the isoelectric point of pH 9.3. As expected, the η increased with protein concentration at all pH values (Figure 3,

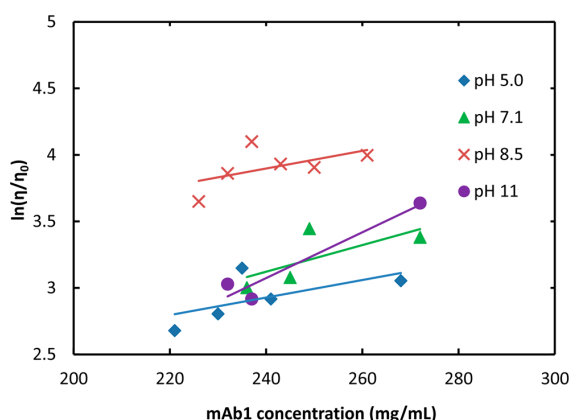


Figure 3. Viscosity of mAb1 solutions as a function of pH at a constant Arg and Glu concentration of 150 mg/mL. The respective Arg and Glu concentrations are 440 and 500 mM at pH 5.0, 470, and 465 mM at pH 7.1, 540, and 380 mM at pH 8.5 and 860 and 0 mM at pH 11.

and Table S4). The η went through a maximum at a pH of 8.5 which is near the pI of mAb1 (9.3). Interestingly, η was similar at pH 7 and 9, two units below and above the pI, respectively, and was even lower at pH 5. Figure 4 shows η and η_{inh} for mAb1 samples containing Arg-HCl instead of Arg-Glu (additional data in Table S5). The η progressively increases from pH 6.4 to 8 and even more at pH 10 where it peaks and then decreases as the pH is raised to 11, as was also observed for the systems with Arg-Glu. The lowest η was obtained at pH 11, although this pH is too alkaline for subcutaneous delivery. The η_{inh} also went through a maximum at pH 10 the value nearest the pI of 9.3.

Table 4. Viscosity, and Turbidity at 350 nm of Large Scale mAb1 Solutions. The Second Row in Each Pair Shown in Bold Is the Result after Sterile Filtration of the Sample in the Row above It

protein concn (mg/mL)	Arg concn (mM)	Glu concn (mM)	pH	η_0 (cP)	η (cP)	η_{inh} (mL/g)	normalized turbidity (mL g ⁻¹ cm ⁻¹)
283 $\pm$ 15	150	170	5.5	1.2	170 $\pm$ 16	17.4	1.8
269 $\pm$ 24	150	170	5.5	1.2	120 $\pm$ 22	17.1	2.6
288 $\pm$ 1.5	450	485	5.5	1.5	83 $\pm$ 23	13.9	1.3
261 $\pm$ 2.2	450	485	5.5	1.5	40 $\pm$ 13	12.6	2.0
288 $\pm$ 19	430	510	5.0	1.5	91 $\pm$ 17	14.3	1.3
269 $\pm$ 7.5	430	510	5.0	1.5	63 $\pm$ 3.9	13.9	1.5

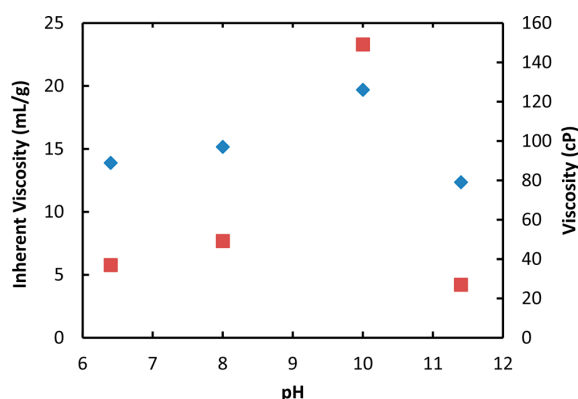


Figure 4. Inherent viscosity (blue diamonds) and viscosity (red squares) for mAb 1 solutions with 860 mM Arg with HCl from Table S5. The viscosity goes through a maximum near the isoelectric point, pH 9.3.

The effect of pH on η was also determined for low cosolute samples as shown in Table 2. The η_{inh} of the low cosolute samples was much higher than that of samples containing Arg-Glu and Arg-HCl over all pH values tested. At a concentration of 200 mg/mL, η for high cosolute samples was as low as ~ 10 cP (Figure 1), which is about 6-fold lower than the low cosolute samples. Because of the high viscosities, the filter permeation decayed rapidly during centrifugation for the low cosolute systems once the mAb concentration approached 200 mg/mL. Therefore, the final concentrations for these samples were only ~ 200 mg/mL as the permeation was too slow to reach the intended target concentration of 250 mg/mL.

Effect of Shear Rate for ~ 250 mg/mL mAb. To further examine the effect of Arg-Glu on the interactions of mAb1, the shear rate dependence of η was measured with a cone and plate rheometer. The sample was prepared by combining samples from runs in four separate 0.5 mL centrifuge filters together. Shear thinning was observed for the mAb1 solution in 20 mM His-HCl with 0.05% Tween 80 as η decreased over 2 orders of magnitude with a shear rate ranging from 0.1 to 1000 s^{-1} . mAb solutions are routinely observed to shear thin due to the presence of mAb networks formed due to associative forces and molecular entanglement.^{8,9,15,22} Tween-80 was added at a concentration well above its critical micellar concentration (CMC) to saturate the air–water interface and prevent the complication of a viscoelastic protein layer on the interface and measure bulk solution rheology.⁵⁶ In contrast, the solution containing 450 mM Arg and 485 mM Glu was nearly Newtonian and underwent very little shear thinning over the same shear rate range, despite the lack of surfactant. This result suggests protein networks were not present in the Arg-Glu solution, consistent with previous results by SLS.²¹ The η values obtained from the syringe viscometer for these same samples are also plotted in Figure 5 at the shear rate determined from the syringe viscometer parameters assuming Newtonian flow. For both samples η values obtained by the syringe viscometer are $\sim 10\%$ higher than those from the cone and plate rheometer and are shown in Table S6.

For polyclonal sheep IgG, the viscosities were well below those for mAb1, down to 17 cP at 258 mg/mL (Table S7). Increasing the concentration of Arg from 430 to 625 mM did

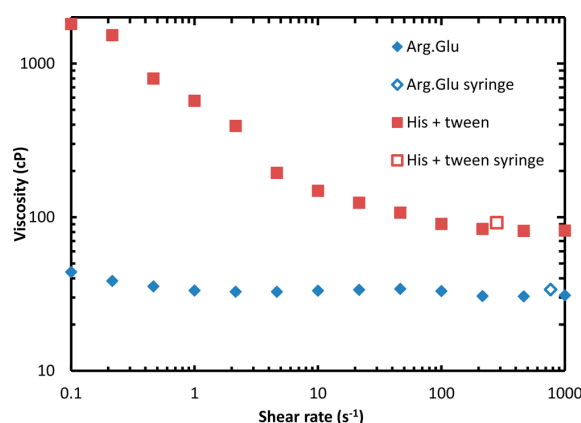


Figure 5. Effect of shear rate on viscosity of mAb1 solutions, one at 228 mg/mL with 20 mM His and 0.05% tween 80 (red squares) and 269 mg/mL with 450 mM Arg and 485 mM Glu (blue diamonds), both at pH 5.5. Hollow symbols were measured by syringe viscometer while full symbols were measured by cone and plate rheometer.

not seem to further lower η . The viscosity was 3 $\times$ lower than previously reported for this protein at a similar concentration without cosolute, a reduction similar to that observed for mAb1.³¹

Effect of a Preferentially Excluded Cosolute Trehalose on Viscosity. To place the results with Arg in perspective, Tre was chosen as a nonelectrolyte cosolute without acidic or basic sites. Table 5 displays results of η and η_{inh} for mAb1 solutions

Table 5. Viscosity of mAb1 Solutions with Tre. All Samples Were Formulated in 50 mM Phosphate Buffer To Set the pH

Protein concn (mg/mL)	Tre concn (mM)	(NH ₄) ₂ SO ₄ concn (mM)	pH	η_0 (cP)	η (cP)	η_{inh} (mL/g)
229	530	0	6.4	1.4	120 $\pm$ 57	19.3
200	530	0	7.2	1.4	130 $\pm$ 11	22.8
246	530	0	8.2	1.4	130 $\pm$ 5.8	18.5
229	185	0	8.2	1.2	160 $\pm$ 28	21.4
228	200	25.0	8.2	1.4	99 $\pm$ 3.0	18.7
243	200	100	8.2	1.4	140 $\pm$ 26	18.9

containing Tre with 50 mM phosphate buffers at pH values ranging from 6.4 to 8, with and without (NH₄)₂SO₄. As shown in Table 5, the addition of Tre does not reduce η_{inh} significantly compared to the low cosolute controls in Table 2. This suggests that Tre does not weaken the attractive specific PPI for mAb1 sufficiently to break up reversible protein aggregates. For systems with added ammonium sulfate, the charge screening along with the trehalose did not decrease mAb1 viscosity either.

The effects of Tre on mAb1 solutions were further tested by combining it with high concentrations of Arg and Glu. Table S8 shows the effect of adding Tre to an Arg-Glu solution at a constant ratio of Tre to Arg-Glu. The η decreases by 45% at ~ 250 mg/mL mAb1 as the total concentration of cosolutes is increased from 380 to 1005 mM. The Tre/Arg-Glu systems have similar values of η compared to the samples containing only Arg-Glu at similar concentrations, suggesting that the Arg-Glu is reducing the viscosity independently of the Tre. The viscosities for these samples are much lower than those containing Tre alone (Table 6) at a given concentration.

Table 6. SEC for mAb1 Solutions Stored for 8 Weeks^a

week	frozen storage at -40 °C	refrigerated storage at 4 °C	room temperature storage at 25 °C
0	99.86	99.86	99.86
1	99.86	99.87	99.86
1.5	99.87	99.90	99.84
2	99.85	99.91	99.83
4	99.77	99.86	
8	99.90	99.88	99.75

^aThe values in the table are the % monomer in the sample measured by the area under the curve for the SEC. The ~250 mg/mL dispersion contained 465 mM Arg-Glu (pH 7.1) and was 99.89% monomer pre-storage.

Colloidal Stability of mAb upon Storage and Dilution.

Formulating mAb1 at high concentration (>250 mg/mL) with 450 mM Arg and 485 mM Glu at pH 5.5 did not produce any irreversible aggregates upon dilution to 1 mg/mL as determined by both size exclusion chromatography (SEC) and dynamic light scattering (DLS), as shown in Table S9. The SEC trace of the original material without any processing except for dilution before running SEC indicated >99.8% monomer. For the >250 mg/mL solution containing Arg-Glu the % monomer was almost identical to that of the starting material after dilution (Figure S4). A brief study of the colloidal stability of mAb1 at high concentration was also conducted by storing a high cosolute sample, containing 250 mg/mL mAb1 with 465 mM Arg and Glu, at -40 °C, 4 °C, and room temperature (25 °C) for 8 weeks as shown in Table 6. The samples stored under all three conditions were seen to be stable with no significant decrease in the % monomer over the course of 8 weeks. Since this formulation has been shown to reduce viscosity, likely by weakening PPI, it is unsurprising that it is resistant to aggregation during storage.⁴³ Further testing of conformational and chemical stability is beyond the scope of this work.

DISCUSSION

mAb Solutions without Cosolute. Several studies have shown with techniques including small-angle X-ray scattering (SAXS), small angle neutron scattering (SANS), static light scattering (SLS), and neutron spin echo (NSE), as well as coarse-grained simulations, that protein self-association and oligomerization caused by attractive interactions may cause a marked increase in η .^{4,12,16,18} Since the void volume in oligomers increases the volume fraction of the mAb, disrupting the oligomer structure can potentially lower η .^{16,57} For a strongly interacting mAb which dimerizes, the addition of 200 mM NaCl was shown to lower η 5-fold from 300 cP to 60 cP at 150 mg/mL by breaking up dimers, as shown by NSE measurements of protein diffusion.¹⁶ The oligomers may be disrupted by screening of the anisotropic attractive electrostatic interactions between local charges and dipoles.^{8,9,22,23} As shown in Table 2, η for mAb1 was seen to decrease by approximately 2-fold upon the addition of 150 mM NaCl to a mAb1 solution in 30 mM His-HCl buffer. However, depending on whether the net or the localized electrostatic interactions are dominant for determining η , the addition of salt can lower,^{14,15,22,58,59} not influence,^{6,16} or in some cases even raise¹⁸ η depending on the particular mAb in question.

Protein interactions may be characterized at low concentrations in terms of a second virial coefficient (B_2) or a diffusion

interaction parameter (k_D). As these parameters become negative, attractive interactions may lead to protein self-association. Connolly et al. found that for a series of mAbs in a given buffer, a direct correlation was observed between η at high concentration with either B_2 or k_D both determined at low concentration, whereby η increased with an increase in attraction.^{7,10} The slightly positive k_D for mAb1 (control with no added cosolute) at pH 6.0 in 30 mM His-HCl buffer (Table S10) is in the middle of Connolly's range. Thus, the observed relatively high $\eta > 100$ cP in Table 2 is consistent with the measured k_D .⁷ Since high η s were seen for mAb1 solutions with low cosolute over a variety of pH values in Table 2, the change in protein-protein interactions with pH over this range appeared to be limited.

Modification of Electrostatic Interactions between mAbs by Arg To Lower η . Molecular dynamic simulations have shown Arg binds to protein surfaces as Arg-protein hydrogen bonds are enthalpically favorable to water-protein H-bonds; Arg forms cation- π interactions with aromatic residues and aids in salt bridge formation. This combination of interactions allows for Arg to interact and bind with numerous sites on many proteins.^{40,60-63} In addition to simulations, Arg-protein preferential interactions^{39,64} have been observed for various proteins via vapor pressure osmometry,⁴¹ high resolution X-ray analysis,⁶⁵ interaction chromatography,^{29,30,66} and static light scattering (SLS),²¹ often resulting in reduced protein aggregation.^{20,21,39,42-44} A recent study by Scherer used SLS to demonstrate direct binding of Arg to an IgG1 antibody, similar to mAb1.²¹ This direct binding weakened protein self-association, removing dimers and oligomers with increasing Arg concentration, with complete multimer removal at 600 mM.²¹ These data are consistent with a recent SAXS study that showed the addition of Arg-HCl or Arg-Asp to a mAb solution reduces protein self-association measured by the apparent radius of gyration (R_g^{app}) and maximum dimension (D_{max}^{app}). These reductions in protein self-association were correlated with reductions in viscosity.⁴⁶ Finally, molecular dynamics simulations have shown Arg-Glu binds more tightly to protein surfaces than Arg-HCl due to hydrogen bonding between Arg and Glu,⁴⁵ which has been suggested as the reason why Arg-Glu reduces mAb aggregation in many systems;⁴²⁻⁴⁴ however, both Arg-Glu and Arg-HCl have been shown to yield similarly low viscosities for mAb1.

At physiological pH, the carboxylic acid group of Arg is negatively charged while its amino and guanidyl groups are positively charged giving it a net +1 charge. Therefore, at physiological pH, bound Arg can increase the magnitude of positive charge on positively charged sites, lend a positive charge to neutral sites and neutralize negatively charged sites. As a result, there will be fewer interactions between oppositely charged local sites, particularly for IgG1 antibodies between positively charged Fc and negatively charged Fab regions.^{4,12,13} Reducing these interactions leads to weaker attraction, which may explain the lowered η at pH 5 to 7 as seen for both Arg-HCl and Arg-Glu, in agreement with the SLS study above.²¹ For arginine, this scenario is supported by an elevated second virial coefficient measured by self-interaction chromatography⁶⁶ and weakened protein binding to ion-exchange columns.^{29,30} The direct binding of Arg to charged sites on a mAb²¹ could be partially responsible for the much greater decrease in η seen for the solutions containing increased Arg (Table 3) compared to those containing NaCl (Table 2). At pH values above 8 where

Arg has no net charge, the protein charge modification by Arg will be lessened along with the effect of ionic strength. Consequently, an increase in anisotropic electrostatic interactions would be expected to increase η at pH 8.5 as observed in Figures 3 and 4. Arg starts becoming negatively charged along with the protein at pH > 11 as the guanidyl group becomes deprotonated. Here bound Arg will make the protein surface more negative and thus weaken electrostatic attraction, which would be expected to lower η as is observed in Figures 3 and 4.

The ionic screening of Arg-Glu may explain why the k_D value for mAb1 decreases upon the addition of high concentrations of Arg-Glu relative to the low cosolute control (Table S10). As mAb solutions have both attractive and repulsive interactions, and the magnitude of these interactions is dependent upon the length scale of interaction which cannot be accounted for in a k_D measurement, which simply measures one number for net interaction strength.⁶⁷ Because of the large protein–protein separation at the low mAb concentrations encountered in the k_D measurements, the anisotropic electrostatic and dipolar interactions will be less significant than global electrostatic repulsion, and therefore the dominant effect of increased ionic screening will weaken repulsive interactions for these measurements. In contrast, at high mAb concentrations, when the average surface-to-surface distance of the mAbs is much smaller, the dominant effect will be short-ranged attractive (electrostatic and hydrophobic) interactions and therefore the addition of Arg-Glu will weaken these interactions and reduce viscosity. Therefore, k_D may not be a good predictive measure of viscosity for systems of varying ionic strength, as has been suggested for B_2 by other cosolute studies.⁴⁷

Modification of Hydrophobic Interactions between mAbs by Salt and Arg To Lower η . In addition to screening anisotropic electrostatic attraction Arg also screens hydrophobic interactions as evidenced by reduced protein binding to hydrophobic columns in the presence of Arg,^{29,30} as well as by increased solubilities of aromatic amino acids.³⁹ Arg is able to interact with hydrophobic residues via its guanidyl group through cation– π interactions.^{39,40,60,61,68} Guanidinium chloride (Gdm-HCl) which is basic when uncharged ($pK_a = 13.6$) denatures proteins at much higher concentrations (>5 M).^{69,70} Unlike guanidine, Arg binding to hydrophobic sites is much weaker because of the carboxylic acid group, resulting in much less unfolding.^{62,63} Additionally, simulations of Arg-HCl at high concentrations show that three Arg ions tend to stack on top of each other with aligned guanidinium groups allowing for the ethylene groups to form a hydrophobic patch which binds to hydrophobic patches on proteins.^{40,61,63,66} Finally, as the concentration of Arg is increased, a greater number of hydrophobic and charged patches can be blocked leading to weaker interactions and therefore lower η explaining the observed trends in Figure 2 and Table 3.

CONCLUSIONS

The viscosity of concentrated ~250 mg/mL mAb1 solutions decreased by up to 6-fold upon the addition of high concentrations of Arg-Glu or Arg-HCl compared to a control in His-HCl buffer at the same pH of 5.5. At pH 5.5 (fixed ratio of Arg-Glu), the viscosity was as low as 30 cP at 263 mg/mL mAb1 (for a η_{inh} of 11.4 mL/g, compared to 20.6 mL/g with low cosolute). A similar reduction in viscosity was seen for Arg-HCl suggesting that $ArgH^+$ played a more important role than the counterion. The viscosities exhibited a maximum near the

pI of 9.3 and decreased significantly as the pH was lowered to 5 or raised to 11. Direct binding of Arg with the mAb, as has been characterized by static light scattering for an IgG1,²¹ may decrease the viscosity by weakening the local anisotropic electrostatic attraction and hydrophobic attraction, which reduces protein self-association and networks. This mechanism is supported by the observed decrease in $[\eta]$ and shear thinning behavior from the addition of Arg-Glu.^{18,21,22,25,29,30,66} In contrast the viscosity did not decrease with the addition of trehalose, which is preferentially excluded from proteins, and it decreased only a small amount for NaCl. Upon dilution in buffer, the percent of protein monomer was >99.75% according to SEC for all storage conditions, indicating that the Arg-Glu cosolute system did not produce irreversible aggregates during storage over 8 weeks. For a polyclonal sheep IgG mixture at 258 mg/mL, a very low η of 17 cP was achieved with the addition of Arg-Glu, approximately 3-fold lower than the low cosolute control for this protein from a previous study.³¹

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.iecr.6b02042.

Donnan equilibrium describing why the final cosolute concentrations are similar to the solvent used to make the samples for the majority of data; figures describing the calibration of the syringe viscometer; detailed data tables from figures, and additional replicate data; figures showing sample turbidities and SEC chromatograms; k_D analysis from dynamic light scattering (PDF)

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Notes

The authors declare no competing financial interest.

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