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Viscosity-Lowering Effect of Amino Acids and Salts on Highly Concentrated Solutions of Two IgG1 Monoclonal Antibodies

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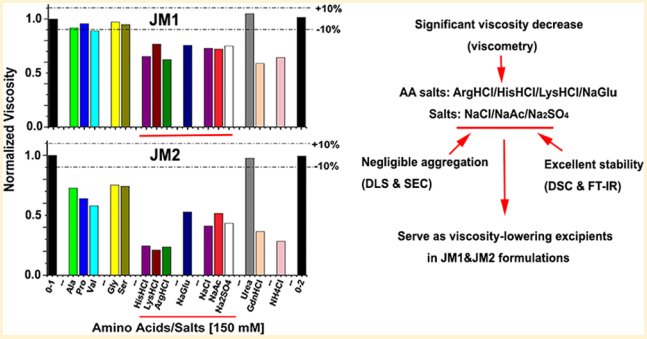
Supporting Information

ABSTRACT: Monoclonal antibodies display complicated solution properties in highly concentrated (>100 mg/mL) formulations, such as high viscosity, high aggregation propensity, and low stability, among others, originating from protein−protein interactions within the colloidal protein solution. These properties severely hinder the successful development of high-concentration mAb solution for subcutaneous injection. We hereby investigated the effects of several small-molecule excipients with diverse biophysical-chemical properties on the viscosity, aggregation propensity, and stability on two model IgG1 (JM1 and JM2) mAb formulations. These excipients include nine amino acids or their salt forms (Ala, Pro, Val, Gly, Ser, HisHCl, LysHCl, ArgHCl, and NaGlu), four representative salts (NaCl, NaAc, Na_2SO_4, and NH_4Cl), and two chaotropic reagents (urea and GdnHCl). With only salts or amino acids in their salt-forms, significant decrease in viscosity was observed for JM1 (by up to 30−40%) and JM2 (by up to 50−80%) formulations, suggesting charge−charge interaction between the mAbs dictates the high viscosity of these mAbs formulations. Most of these viscosity-lowering excipients did not induce substantial protein aggregation or changes in the secondary structure of the mAbs, as evidenced by HPLC-SEC, DSC, and FT-IR analysis, even in the absence of common protein stabilizers such as sugars and surfactants. Therefore, amino acids in their salt-forms and several common salts, such as ArgHCl, HisHCl, LysHCl, NaCl, Na_2SO_4, and NaAc, could potentially serve as viscosity-lowering excipients during high-concentration mAb formulation development.



KEYWORDS: *monoclonal antibody, viscosity, subcutaneous injection, amino acids, protein−protein interaction, mAb stability*

INTRODUCTION

Over the past 20 years, monoclonal antibodies (mAbs) have rapidly evolved into a major class of highly promising therapeutic molecules against various diseases, such as inflammatory diseases, cancers, and other human disorders,[1−3] largely due to their superb target specificity and safety profile. As biologics with large size and complex structure, the formulation and delivery of mAbs pose substantial challenges.[4] Although current mAb therapeutics are mostly administered through intravenous infusion due to their high doses, subcutaneous injection of mAb solution or suspension endows more patient compliance and thus is more desirable.[5−8] However, subcutaneous delivery of mAbs is often restricted by the complicated solution properties of mAb at high concentrations of above 100 mg/mL. These properties, including solubility, viscosity, and aggregation, are governed by abundant protein−protein interactions.[9] The protein−protein and protein−excipient interaction under various environmental conditions have been investigated to facilitate the formulation design of the biopharmaceuticals.[10]

Although protein engineering enables editing and optimization of the intrinsic biophysical properties of mAbs, formulation optimization is the most cost-efficient and effective approach to improve the mAb solution properties, especially at the product development stage where the amino acid sequence of the mAb has been selected. Excipients, such as sugars/polyols, amino acids (AAs), polymers, and salts, are added to stabilize protein therapeutics in the formulation strategy.[11−13] Previously, investigations were conducted on the effects of excipients (pH-buffering reagents, mannitol, sucrose, arginine, proline, arginine glutamate, urea, guanidine-HCl, NaCl, and other salt

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ions) on the solubility,[14] aggregation,[10,15] conformational and storage stability[16−22] of mAbs in solution, solid state, or during drying.[5] More recently, the effects of different excipients on the viscosity of highly concentrated protein (especially, mAb solutions at >100 mg/mL) have been investigated. It was observed that short- and long-range interactions, including the hydrophobic interaction,[23] dipole−dipole interaction,[24] and charge−charge interaction due to heterogeneous surface charge distribution,[25,26] can all greatly influence the mAb self-association and the viscosity of mAb solutions. Sugars such as sucrose and mannitol are often used as stabilizers in the protein formulation. He et al. systematically studied the effect of sugar molecules on the viscosity of two mAb (one IgG1 and one IgG2) liquid formulations.[27] It is found that the seven sugars used in the study, including disaccharides and monosaccharides, all significantly increased the viscosity of mAb solutions especially at high protein concentrations. AAs, such as arginine and proline, can also be used as stabilizers in protein formulations to increase solubility,[28] reduce aggregation,[29,30] decrease viscosity,[31,32] and enhance stability.[21,33]

The viscosity of highly concentrated mAb solution is presumably caused by the specific or nonspecific interactions (i.e., H-bonding, hydrophobic, ionic, electrostatic, dipole−dipole, etc.), which is hard to characterize without the molecular details and the three-dimensional structure. AAs with their diverse side chains have various biophysical−chemical properties. By observing and comparing their effects on mAb solution properties, they could serve as probes to elucidate the interaction mechanisms between mAb molecules in the high-concentration solution. Therefore, we hereby investigated the viscosity-lowering effect of various natural AAs and representative salts on two IgG1 formulations, and we evaluated the impact of these excipients on the conformational stability of both mAbs. Furthermore, we attempted to propose some potential mechanisms governing the high viscosity of these two highly concentrated mAbs.

MATERIALS AND METHODS

Materials. Two IgG1 mAbs were kindly provided by Janssen Research & Development (Johnson & Johnson, Shanghai, China) and designated by us herein as JM1 and JM2. The solution of both mAbs was exchanged to a 20 mM Histidine-HCl (HisHCl) buffer with a pH of 6.0, using a desalting column and/or through dialysis. This buffer was treated as the primary buffer condition unless specifically mentioned. Reagents for preparing AAs and salts excipients were of analytical grades and obtained from commercial vendors as follows: L-glycine (Sinopharm), L-alanine (Sigma), L-valine (Sinopharm), L-proline (Sigma), L-serine (Sinopharm), L-histidine (J&K chemical), L-arginine-HCl (Sigma), L-histidine-HCl (J&K chemical), L-lysine-HCl (Amresco), L-glutamic sodium (Sinopharm), guanidine-HCl (Amresco), urea (Amresco), NaCl (Sinopharm), NaAc (Sinopharm), Na_2SO_4 (Sinopharm), NH_4Cl (Sinopharm).

Solution Preparation. All buffers were filtered through 0.2 μm nitrocellulose membranes before usage (Millex, Merck Millipore Ltd., Ireland). Buffer exchange of mAb solutions was realized with dialysis, desalting column (Hiprep, GE Healthcare) on FPLC or centrifugal filter units with the molecular weight cutoff of 30 kDa (Amicon Ultra, Merck Millipore Ltd., Germany). Samples were concentrated using centrifugal filter units.

Concentration Measurement. The concentration of mAbs in solution was determined with UV−vis spectrophotometer (UV-4802, UNICO, Shanghai, China) by measuring the UV absorbance at 280 nm with diluted solutions and calculated with the respective extinction coefficient of the mAbs.

Viscosity Measurement. A microfluidic slit rheometer referred to as microVISC (Rheosense Inc., CA) was employed to measure the steady shear viscosity of the solutions at high shear rates.[34] The specific microfluidic device used in this study, microVISC Type A chipset (14HA05100550), consists of a rectangular cross-section channel (w = 2 mm; d = 50 μm) made out of Pyrex mounted on a gold-coated silicon base containing three flush mounted pressure sensors. The pressure drop, ΔP, required to drive the flow with rate, Q, is related to the wall shear stress, τ_w, by the expression $wd\Delta P = 2L(w + d)\tau_w$, whereas the nominal wall shear rate, γ_w, associated with fully developed laminar flow of a Newtonian fluid is $\gamma_w = 6Q/wd^2$. In a typical experiment, the flow rate, Q, is varied using a syringe pump and a 400 μL disposable pipet (Rheosense Inc., CA). The microVISC device records the pressure drop as a function of flow rate, and the data is then used to determine the nominal or apparent viscosity $\eta(\gamma) = \tau_w / \gamma_w$.

The fluid temperature throughout the instrument was maintained at desired constant values using thermal jackets. Water was used as reference liquid to ensure the accuracy of the rheometer before each measurement. At least duplicated samples were withdrawn from each formulation and average viscosity values based on 3−5 measurements were recorded.

Dynamic Light Scattering (DLS). The dynamic size and polydispersity of mAb molecules in solution were obtained on a Zetasizer Nano ZS90 dynamic light scattering (DLS) machine (Malvern Instruments Ltd.,Worcestershire, U.K.) with a 633 nm red laser for particle size detection. Initially, 100 μL of the mAb solution was first equilibrated at 25 °C for 2 min in a disposable UV-plastic cuvette (Brand GmbH and CoKG, Wertheim, Germany) before each measurement. Each sample was recorded three times, with 16 runs each time.

Zeta potential of each sample was also measured using the same Malvern Zetasizer DLS instrument at 25 °C using DTS 1070 disposable folded capillary cells (Malvern Instruments Ltd.), and 1.2 mL of each sample was prepared in 20 mM HisHCl buffer at pH 6.0 and directly measured in triplicate.

Size-Exclusion Chromatography (SEC). Size-exclusion chromatography was used to detect the monomer loss and aggregation formation using the Shimadzu LC-20AT high-performance liquid chromatography system (HPLC, Kyoto, Japan). Tosoh TSKgel BioAssist G3000SWxl column (7.8 mm × 300 mm) is used together with the TSKgel guard column SwXL (6.0 mm × 40 mm). Twenty micrograms of each sample was loaded onto the column and eluted isocratically at a flow rate of 0.7 mL/min with a mobile phase of 200 mM sodium phosphate at pH 6.0 and room temperature of around 25 °C. The protein concentration was measured by UV absorbance at 280 nm. Area under the curve of peaks in the chromatogram was used to calculate the amount of monomer and aggregates of mAbs.

Differential Scanning Calorimetry (DSC). DSC measurement was performed using a Manual Microcal VP-Capillary DSC system (GE Healthcare, U.S.A.). The samples at 1 mg/mL concentration were filled into the tantalum cells with an active volume of 130 μL, using corresponding buffer as a reference. The thermogram was obtained with a scan from 25

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to 100 °C at a rate of 180 °C/h. All data were analyzed with Origin 7.0 software (OriginLab Corporation, U.S.A.). Thermograms were corrected by subtraction of buffer blank scans and normalization to the protein concentration. Considering the possible native−intermediate−denatured state transition of the multidomain mAb, the thermal transition midpoint (T_m) was determined by fitting the transition curve with a non-two-state model, which gave the least fitting residual error.

Fourier Transform Infrared Spectroscopy (FT-IR). Fourier transform infrared spectroscopy studies were conducted using an FTIR-ATR Vertex 70 spectrometer (Bruker Optics, Ettlingen, Germany) at room temperature (~25 °C). The region of 1300−1800 cm^{-1} in the spectra was baseline corrected and normalized. The mAb solution was deuterated through two rounds of lyophilization of 300 μL of the mAb solution (50 mg/mL) in various formulations and reconstitution in D_2O. The background information from the solution environment was subtracted from that of the mAb formulation using Origin software (OriginLab, Northampton, MA). The region of 1600−1700 cm^{-1} corresponding to the amide I was extracted for analysis.

RESULTS AND DISCUSSION

Concentration Dependence of the Viscosity of JM1 and JM2. We measured the viscosity of JM1 and JM2 at different concentrations, and we compared that with the viscosity of bovine serum albumin (BSA), which serves as a model protein. Both mAbs can be concentrated, resulting in a clear solution of more than 300 mg/mL in 20 mM HisHCl at pH 6.0, suggesting excellent solubility. The viscosity of the three protein solutions all demonstrate exponential dependence on the concentration (Figure 1), which is a characteristic of

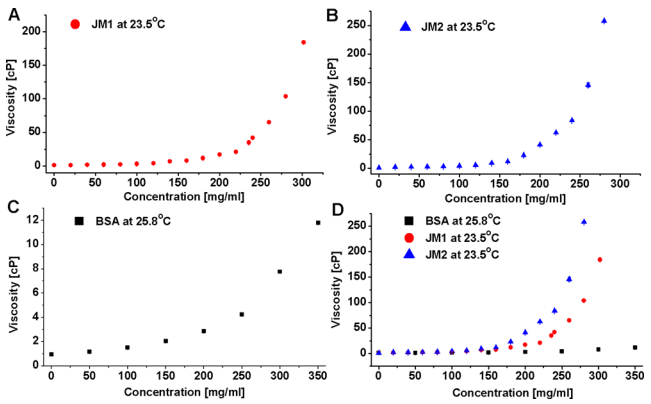

Figure 1. Concentration-dependent viscosity of JM1, JM2, and BSA solutions. The protein samples with concentration series were prepared in a buffer of 20 mM HisHCl at pH 6.0.

non-Newtonian solution. The viscosity of JM1 and JM2 increases sharply as the concentration increases, especially when the concentration is above 150 mg/mL (Figure 1A, B). This is because protein−protein interactions intensify at the high concentration, making the solution behavior far more deviated from that of the dilute Newtonian solution.[35,36] The viscosity of JM2 increases more sharply (~270 cP at 280 mg/mL) than that of JM1 (~110 cP at 280 mg/mL), suggesting stronger protein−protein networks in JM2 solutions. BSA, as nonimmunoglobulin protein and a comparison here, displays quite slow increase in the viscosity as the concentration increases (less than 15 cP at 300 mg/mL, Figure 1C). The differentiation of viscosity−concentration dependence of JM1, JM2, and BSA originates from their intrinsic molecular differences.

Physiochemical Properties of Selected AAs/Salts. Viscous solutions of JM1 and JM2 with high viscosities especially at concentration higher than 160 mg/mL are not suitable for subcutaneous injections. Therefore, here we introduced several natural amino acids and representative salts (Supplementary Figure 1, left panel of chemical structures) as excipients into the mAb solution to decrease the viscosity of highly concentrated JM1 and JM2. The amino acids used can be generally classified into the following groups: hydrophobic (Ala, Pro, Val), slightly hydrophilic but not charged (Gly, Ser), the hydrochloride form of basic amino acids (HisHCl, LysHCl, ArgHCl), the sodium salt of acidic amino acids (NaGlu) and other sodium salts (NaCl, NaAc, Na_2SO_4), chaotropic reagents (Urea, GdnHCl), and an ammonium salt (NH_4Cl). The viscosity of 150 mM AAs/salts in the primary buffer of 20 mM HisHCl at pH 6.0 was obtained at 25 °C. All buffers in the absence or presence of AAs/salts show viscosity of around 0.95−1.10 cP similar to the water viscosity of 0.91 ± 0.02 cP on the same viscosity chip (Supplementary Figure 1, table panel at the right).

Selection of an Effective AAs/Salts Concentration To Compare the Excipient Effects on mAb Viscosity. To select a suitable excipient concentration for further investigation of viscosity−excipient relationship, we first evaluated the viscosity of JM1 and JM2 with increasing concentration of AAs/salts using four representatives: Gly, Pro, ArgHCl, and NaCl (Figure 2). The concentration of AAs/salts varies from 0 up to 300 mM with 50/100 mM intervals. Glycine shows the least effect to decrease the viscosity of JM1 and JM2, where the minimal viscosity of each formulation was reached after the addition of around 50−100 mM Pro/ArgHCl/NaCl and 100−150 mM glycine, respectively. Proline has a relatively stronger viscosity-lowering effect than glycine, after the addition of 100−150 mM into the JM1 and JM2 formulations. In comparison, ArgHCl and NaCl provide a significant viscosity-lowering effect even at the low concentration of 50 mM and the minimum viscosity was obtained when the salt concentration reached 150−300 mM. This phenomenon is also reported on other mAbs by Liu et al.[37] and Connolly et al.[38] Although the viscosities of both JM1 and JM2 decrease upon adding these two salt-form excipients, the extent of viscosity decreasing is slightly different by NaCl or ArgHCl. It is worth noting that the viscosity-lowering effect upon adding NaCl or ArgHCl is believed to be jointly contributed by the properties of the mAb molecules, the chemistry of the salt species, and the ionic strength of the salt. Furthermore, both glycine and proline show viscosity-decreasing effects at low concentration (below 100 mM) and viscosity-increasing effect at high concentration (above 200 mM). This biphasic concentration dependence phenomenon was also observed on Gly, ArgHCl, GdnHCl, LysHCl, and NaCl in bovine gamma globulin solution by Inoue's group, where the excipient concentration are tested up to 1000 mM.[32] This has been attributed to preferential binding (salting-in) at low concentrations and preferential exclusion (salting-out) at high concentrations of additives.[39−41] Considering all the results above, we selected an excipient concentration of 150 mM to investigate the effects of AAs/salts on the viscosity and stability of mAb formulations in this study.

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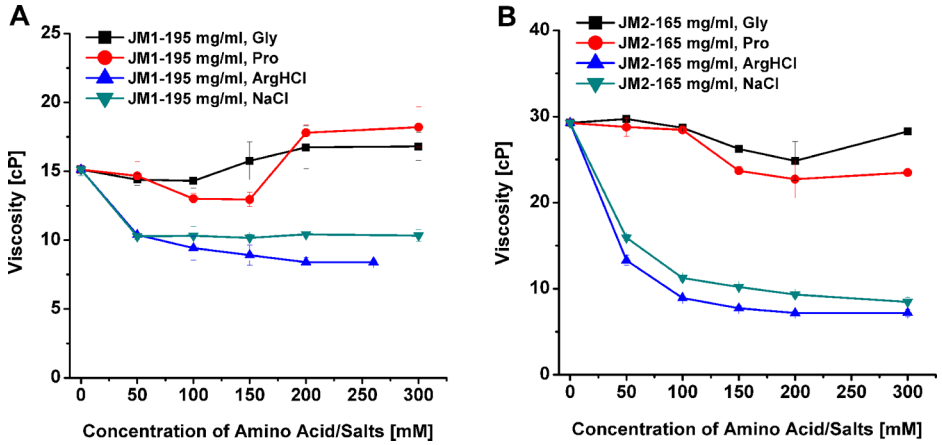

Figure 2. Concentration effect of AAs/salts on the viscosity of JM1 (A) and JM2 (B). JM1 of 195 mg/mL and JM2 of 165 mg/mL were prepared in the primary buffer of 20 mM HisHCl at pH 6.0 in the presence of AAs/salts with concentration series of 0−300 mM.

Effect of AAs/Salts on the Viscosity of JM1 and JM2. The viscosity of JM1 and JM2 formulations in the absence and presence of various AAs/salts were obtained using the microVISC system. To facilitate the analysis, we define a viscosity change of more than 2 cP, or more than 10% upon normalization, as a "significant viscosity change" for samples with viscosity around 15−30 cP. With this standard, it was found that HisHCl, LysHCl, ArgHCl, NaGlu, NaCl, NaAc, Na_2SO_4, GdnHCl, and NH_4Cl (all in salts forms) caused significant viscosity decrease in JM1 formulations, from 18 cP to 11−14 cP (by 4−7 cP or 30−40%) (Figure 3A1, A2). Similarly, salt-form excipients also induced obvious viscosity decrease in JM2 formulations from 28 cP to 7−15 cP (by 13-21 cP or 50−80%, Figure 3B1, B2), and the extent of viscosity decrease was much stronger compared to the corresponding JM1 formulations. All the nonsalt AAs, such as hydrophobic AAs (Ala, Pro, and Val) and hydrophilic AAs (Gly and Ser), produced no or negligible viscosity-decreasing on JM1 (Figure 3A1, A2) but modestly reduced the viscosity of JM2 from 28 cP to 16−21 cP (by 7−12 cP or 25−45%) (Figure 3B1, B2). As shared by other researchers,[37] the noncharged chaotropic reagent urea does not show any viscosity-changing effect on either JM1 or JM2 formulations. Interestingly, all the excipients tested shows very weak viscosity-lowering effect on the model protein BSA (Figure 3C1, C2).

It is quite possible that the salts could provide an electrostatic shielding effect (ion−protein interaction) on the protein surface thus decreasing the solution viscosity by disrupting the protein−protein interaction. The addition of 150 mM of ArgHCl, GdnHCl, LysHCl, and NaCl have a much stronger viscosity-decreasing effect on JM1 and JM2 solution compared to that of the nonsalt-form AAs like Gly. This effect was also observed on bovine gamma globulin solution by Inoue et al.[32] However, the viscosity-lowering effect of ArgHCl, HisHCl, and LysHCl on BSA was found to be not significant in our study, which is in contradiction with previous observations.[31,32] This inconsistency might be related to the difference in BSA solutions, as well as the methodologies to determine the viscosity.[31,32,34,42] Solution viscosity of BSA at high concentrations (e.g., 200, 250, and 300 mg/mL) measured by microfluidic viscometer, Ubbelohde glass capillary viscometer, or literature values are summarized in supplementary Table 1 and supplementary Figure 3. Several factors including shear stress, solution−air, or solution−wall interface interaction, and

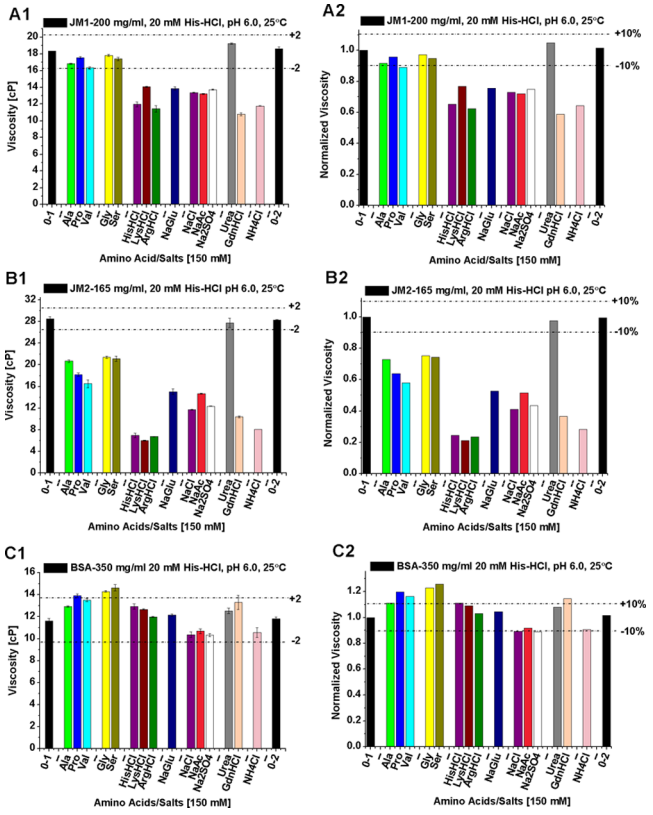

Figure 3. Viscosity of JM1 (A1 and A2), JM2 (B1 and B2), and BSA (C1 and C2) in the presence of 150 mM of AAs/salts. A1, B1, and C1: Viscosity of JM1, JM2, and BSA in the formulation with or without 150 mM AAs/salts as additives; A2, B2, and C2: Normalized averaged viscosity of each mAb formulation with respect to the averaged viscosity of mAbs in the primary buffer (20 mM HisHCl, pH 6.0, no AAs/salts, indicated as 0−1 and 0−2). The concentration of JM1, JM2, and BSA are of 200, 165, and ∼350 mg/mL, respectively.

solvent evaporation during measurement process (especially for small volume), might have contributed to the variation in the final measured viscosity. Kosmotropic ions (water structure maker, such as anionic SO_4^{2-} and cationic NH_4^+ used in this study) and chaotropic reagents (water structure breaker, such as urea and GdnHCl used in this study) do not affect the viscosity of the mAb formulations oppositely, despite their opposite kosmotropic/chaotropic properties, suggesting the

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low probability that these reagents affect the viscosity of mAb formulations purely through mediating the protein−water and water−water interactions. Instead of the simple protein−water interaction mechanism, a sound explanation is that the mAb−excipient interaction is mostly through the charge−charge interaction, because only GdnHCl salt but not neutral urea significantly decreased the viscosity of the JM1 and JM2 formulations, although both excipients are chaotropic reagents. Kanai et al. also reported a similar finding where GdnHCl but not urea reduced the viscosity of a highly concentrated mAb (IgG1) solution, and the mAb interaction was believed to be mediated by the Fab−Fab interaction.[43] Hydrophobic salts such as Ala, Pro, and Val also slightly reduced the viscosity of JM2, possibly by breaking the hydrophobic interactions between JM2 molecules. Guo Z. et al. also found that a wide range of hydrophobic, bulky, and aliphatic salts could reduce the viscosity of mAb formulations.[23] The viscosity of JM1 formulation decreased little upon the addition of hydrophobic (Ala, Pro, and Val) or hydrophilic AAs (Gly and Ser), whereas the viscosity of JM2 formulation decreased with the addition of either nonsalt or salt-form excipients, suggesting the contribution of mAb-dependent interaction mechanism to the mAb formulation viscosity. Most probably, the viscosity of JM1 could be induced by charge−charge (or dipole−dipole) interaction, whereas that of JM2 could be induced by a multiple protein−protein interaction mechanisms, including a dominant charge−charge (or dipole−dipole) interaction and a secondary hydrophilic/hydrophobic interaction. The addition of 150 mM salts or salt-form AAs caused greater reduction in viscosity of JM2 (e.g., from 28 to 7 cP with ArgHCl) than that of JM1 (e.g., from 18 to 11−12 cP with ArgHCl), again indicating a mAb-dependent viscosity-lowering mechanism. Besides, although bovine/human serum albumin (BSA/HSA) could serve as model proteins to elucidate some general properties of protein solution,[31,34] their relatively low viscosity (Figure 1C) and weak responses toward the addition of various excipients (Figure 3C) indicate that it may not be an appropriate model protein for the investigation of the viscosity of mAb formulations.

Is the Viscosity Caused by the Charge−Charge Interaction from the Net Charges of the mAbs? The most significant viscosity decrease was caused mainly by salt-form additives, suggesting that the charge interaction could be the dominant mechanism causing the high viscosity of mAb formulations. Therefore, we measured the zeta potential of JM1, JM2, and BSA in the primary buffer of 20 mM HisHCl at pH 6.0 with two concentrations of 20 and 200 mg/mL (Table 1). The zeta potential of JM1 (+2.8 mV), JM2 (+4 mV), and BSA (−2.5 mV) is not significantly affected by the protein concentration. If the net charges dominated the charge−charge interaction, then JM2, with more positive net charges, should have a smaller viscosity than JM1, because a stronger repulsive force is expected to reduce the viscosity. However, the reality is that JM2 is more viscous than JM1 at the same high concentration (Figure 1D). A plausible explanation for this discrepancy could be that the charge−charge (or dipole−dipole) interaction between the mAbs is mostly controlled by the heterogeneous charge distribution on the mAb molecules surface, rather than by the net charges of the mAbs.[25,41] In other words, the mAb molecules may have dipole or even multipole to cause intermolecular attractions.[24]

Table 1. Zeta Potential of JM1, JM2, and BSA in 20 mM HisHCl, pH 6.0

	zeta potential [mV]	
protein solution	20 mg/mL	200 mg/mL
JM1	2.8 ± 0.325	2.78 ± 0.04
JM2	4.64 ± 0.658	4.4 ± 0.368
BSA	−2.76 ± 0.13	−2.16 ± 0.275

Is the Viscosity Induced by the Intermolecular Interaction of Aromatic Groups? Three-dimensional (3D) excitation−emission-intensity plot obtained by fluorescence spectroscopy measurements can serve as a fingerprint of the physical−chemical environment of intrinsic aromatic amino acids such as tryptophan, tyrosine, and phenylalanine in protein.[44] Here we conclude that the viscosity-lowering effect is unlikely achieved by the interaction between the excipients and the intrinsic aromatic groups of the mAbs, because there is no obvious change on the 3D excitation−emission-intensity plot of JM1 and JM2 formulations in the absence and presence of various AAs/salts (Supplementary Figure 2). Only Val induced changes on the 3D fluorescence plot, probably due to the highly hydrophobic environment it provided to the mAb solutions compared with other excipients tested.

Effect of AAs/Salts on the Aggregation of JM1 and JM2 during Storage. As a common risk in protein drug formulation, aggregation may compromise the safety and efficacy of mAb therapeutics, which have been vigorously assessed and analyzed during the mAb production, formulation and storage.[11,12,45,46] Thus, to evaluate the aggregation profile, samples of JM1 and JM2 with multiple AAs/salts stored at 4 and 40 °C (stressed condition) are subjected to HPLC-SEC analysis. Before the SEC experiment, JM1 of 200 mg/mL and JM2 of 165 mg/mL were preincubated at 4 and 40 °C for 14 days and then stored at −40 °C. Adding 150 mM AAs/salts directly to the formulation of JM1 (200 mg/mL) and JM2 (165 mg/mL) does not induce obvious aggregation as evidenced in Figure 4 (A1, A3, B1, B3, and black square in A5, A6, B5, B6). The decrease of monomer recovery (Figure 4A5, B5) was caused by the loss of mAbs due to the precipitation of large aggregation presumably induced by excipient addition, by incubation under 4 or 40 °C, or by the freeze−thaw process. The reduction of monomer recovery (Figure 4A5, A6) reflects the sum of soluble aggregation and the amount of degradation within the samples loaded onto the SEC column. All JM1 and JM2 formulations demonstrate more than 90% monomer recovery compared to the ones that was stored at 4 °C for 2 weeks without any additives. In comparison, several formulations show significant monomer loss (monomer recovery of less than 80%) after 40 °C storage for 2 weeks due to precipitation, including JM1 formulation in the primary buffer without additives or with GdnHCl (Figure 4A2, A4, and red dots in A5), and JM2 formulations in primary buffer without additives or with Val, urea, or GdnHCl (Figure 4B2, B4, and red dots B5). After removing precipitates, most JM1 and JM2 formulations have the monomer portion values on column of more than 95%. Several formulations contain more than 20% of soluble aggregation or denaturation, including JM1 in primary buffer without additives or with Val, Urea, or GdnHCl (Figure 4A2, A4, and red dots in A6) and JM2 in valine (Figure 4B2, B4, and red dots in B6). Overall, with a few exceptions (such as GdnHCl), these salt-form excipients only induce negligible mAb aggregation and furthermore, the addition of them appear to improve the mAb formulation stability. Combining the

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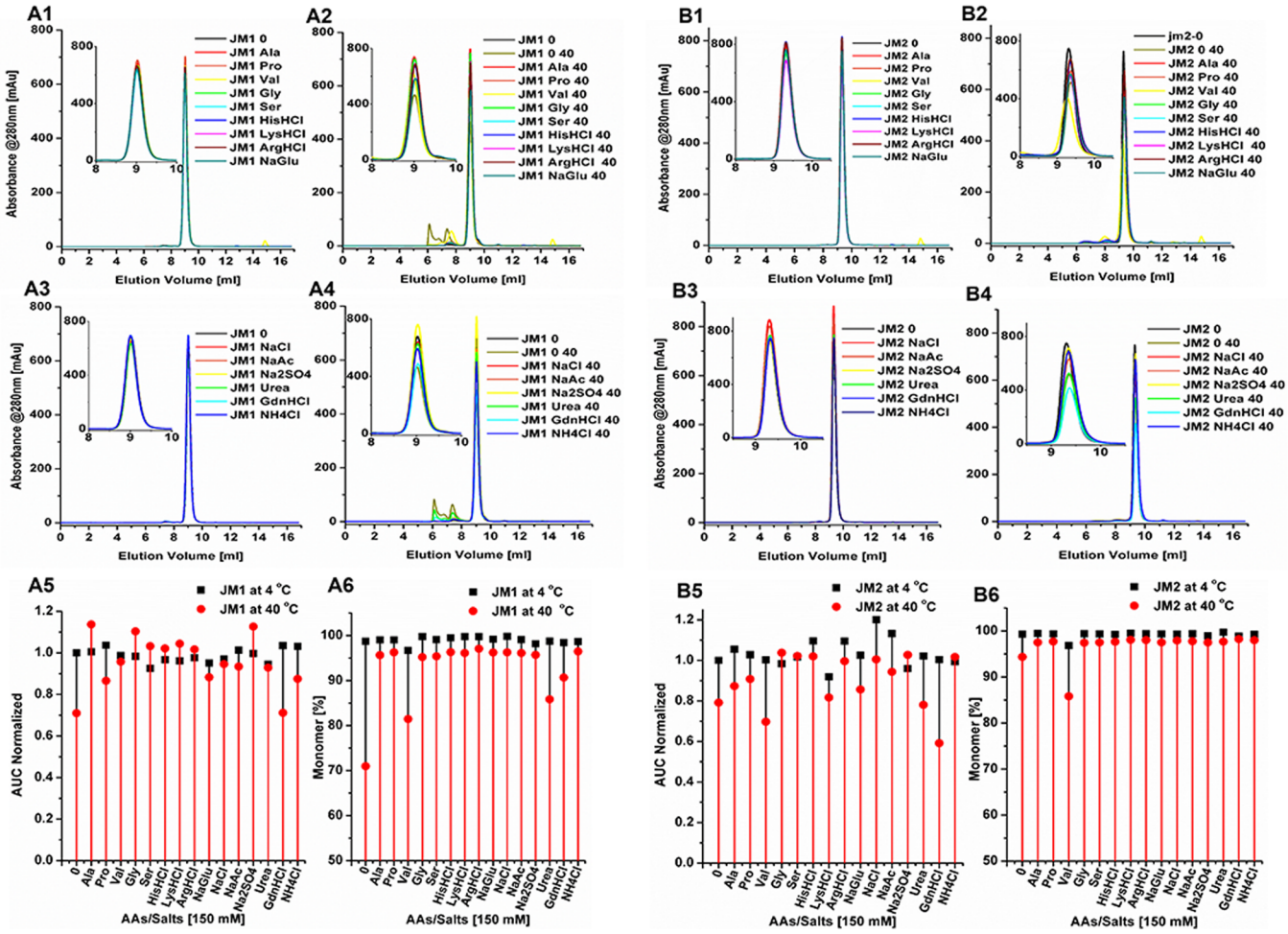

Figure 4. SEC profile of JM1 (A1−6) and JM2 (B1−6) in formulation with various excipients. (A1 and A3) JM1 incubated at 4 °C in 150 mM AAs and salts, respectively. (A2 and A4) JM1 incubated at 40 °C in 150 mM AAs and salts, respectively. (A5) The line-drop plot of area under curve (AUC) of monomer peaks of JM1 in various formulations with 150 mM additives normalized with that in a formulation without additives incubated at 4 °C in the solution of 20 mM HisHCl at pH 6.0. (A6) The line-drop plot of monomer recovery percentage in each formulation of JM1 of 200 mg/mL stored at 4 and 40 °C in the solution of 20 mM HisHCl at pH 6.0 with and without additives. The same condition and order apply to the right panel (B1−B6) for JM2 formulations.

aforementioned viscosity-lowering effect, these salt-form excipients could potentially serve as viscosity-lowering stabilizers in JM1 and JM2 formulations, thus making subcutaneous injection of high-concentration mAb formulations feasible. Hydrophobic amino acids, such as proline,[33] leucine,[30,47] and isoleucine,[33] were reported previously to be able to reduce the aggregation of intravenous immunoglobulins during storage and were therefore proposed as possible alternatives for sugars to be included in IgG formulations. However, our data with valine suggests that it is unlikely an ideal protein stabilizer for JM1 and JM2 formulations, as evidenced with the aggregation profile and the temperature induced conformational transition profiles (discussed in the following sections).

Hydrodynamic Diameters of JM1 and JM2 in the Formulations. The hydrodynamic diameters and size distribution of JM1 and JM2 in formulations were measured by DLS to detect any potential mAb aggregations (Table 2). The 10 mg/mL mAb solutions subjected to DLS measurement were prepared by dilution of highly concentrated protein formulations (JM1 of 200 mg/mL and JM2 of 165 mg/mL) previously stored at 4 °C for 2 months. The small hydrodynamic diameters of around 12−13 nm and the low polydispersity of less than 20% suggest that the mAb molecules predominantly exist as monodispersive monomers in the diluted JM1 and JM2 formulations.

Effect of AAs/Salts on the Conformational Stability of JM1 and JM2. The conformational stability of JM1 and JM2 were examined in the absence and presence of 150 mM AAs/salts by DSC (Figure 5A1, A2, B1, and B2). JM1 displays three endothermic unfolding events with respective transition temperature of T_m1, T_m2, and T_m3 (Figure 5A3). The first, second, and third transition correspond to the unfolding of the C_H2, Fab, and C_H3 domain, respectively.[48−50] JM2 shows only two thermal unfolding temperatures of T_m1 and T_m2 (Figure 5B3), where the C_H2 domain unfolds at T_m1 and Fab and C_H3 domains unfold at similar temperature around T_m2. The overall DSC profile of each mAb is not significantly affected by the addition of 150 mM AAs/salts, except for small shifts in the transition temperatures (Figure 5A1, A2, B1, and B2). The observed changes in the transition temperatures, ΔT_m in Figure 5A4 and B4, was derived from the corresponding T_m values with 150 mM AAs/salts subtracted by the T_m of the formulation without AAs/salts. Various AAs/salts mostly increase (a positive ΔT_m) or decrease (a negative ΔT_m) the T_ms of JM1 and JM2 very similarly. For example, Ala, Pro, Gly, Ser and NaGlu all slightly increased the thermal stability of JM1

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Table 2. Average Hydrodynamic Size and Size Dispersity of mAb Formulations with/without Various AAs/Salts

	JM1		JM2	
150 mM AA/salt	d_H/[nm]	PDI	d_H/[nm]	PDI
0−1	11.52	0.121	13.95	0.081
Ala	11.57	0.056	14.16	0.139
Pro	11.51	0.065	13.51	0.065
Val	12.47	0.091	13.39	0.097
Gly	11.51	0.048	13.61	0.131
Ser	12.14	0.11	14.12	0.121
HisHCl	12.16	0.123	13.95	0.3
LysHCl	12.33	0.082	12.22	0.136
ArgHCl	11.72	0.085	12.46	0.162
NaGlu	12.57	0.153	13.55	0.111
NaCl	11.62	0.124	12.04	0.12
NaAc	11.89	0.098	12.58	0.066
Na_2SO_4	11.72	0.094	12.42	0.068
urea	11.82	0.07	12.4	0.077
GdnHCl	11.34	0.041	11.93	0.109
NH_4Cl	11.1	0.062	11.26	0.049

Note: The hydrodynamic diameter of mAb is measured with a concentration of 10 mg/mL in 20 mM HisHCl with or without 150 mM AA/Salt at pH 6.0 and 25 °C. JM1 and JM2 were diluted from 200 and 165 mg/mL respectively, which had been used for viscosity measurements and then stored in the above-mentioned buffers at 4 °C for 2 months. d_H: hydrodynamic diameter; PDI: polydispersity index.

and JM2, due to the observed T_m increasing of all domains. In comparison, Na_2SO_4 and urea can partially increase the stability of a certain domain (for example, C_H2 in the presence of urea for both JM1 and JM2), and the decreasing of transition temperature was apparent when Val, HisHCl, GdnHCl, or NH_4Cl was added. All other formulations in the presence of salts or salt-form AAs, including LysHCl, ArgHCl, NaCl, and NaAc, display some slight conformational destabilizing effect evidenced by small T_m decrease. Formulations with increased T_m upon the addition of certain excipients generally showed no or slight decrease in their viscosity, such excipients include Ala, Pro, Gly, Ser, and urea. Formulations with decreased T_m upon the addition of some other excipients usually show viscosity-lowering effects, and such excipients include HisHCl, LysHCl, ArgHCl, NaCl, NaAc, GdnHCl, and NH_4Cl. This phenomenon is conceivable because excipients that decrease the viscosity of mAb formulations would likely interact with mAbs strongly to perturb the intermolecular interactions between the mAb molecules, thus enabling unfolding at a lower temperature. Valine is an exception here due to the fact that it does not significantly decrease the viscosity of JM1 or JM2, yet causes a negative T_m shift. This could be attributed to its unique hydrophobic nature, i.e., the most hydrophobic excipient among all tested excipients in this study. A very hydrophobic reagent has stronger tendency to interact with the relative hydrophobic cores in protein structure, especially when the temperature is increased. Besides Valine, NaGlu and Na_2SO_4 are two more exceptions that do not follow the aforementioned correlation, and the underlying reason is yet to be identified. Although it decreases the viscosity of both JM1 and JM2, NaGlu stabilize the conformation of JM1 and JM2 under increased temperature. The stabilizing effect of NaGlu is also reported on the formulation of granulocyte colony stimulating factor.[51] The underlying interaction mechanism between Na_2SO_4 and mAbs may vary with the mAb molecules, as

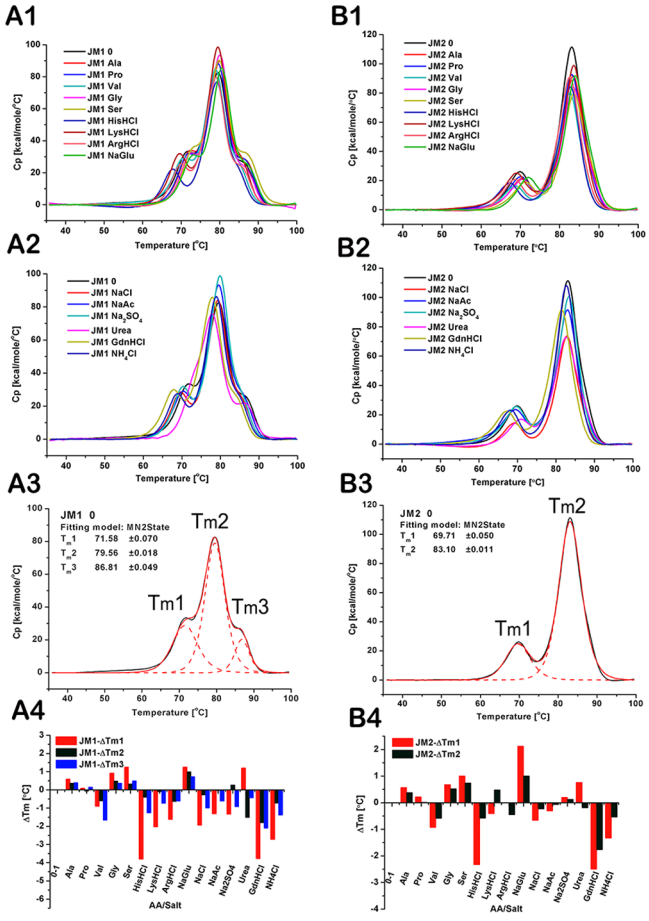

Figure 5. Thermograms of JM1 (A1−4) and JM2 (B1−4) in formulations with various excipients. (A1 and A2) Overlay of DSC curves of JM1 formulations with AAs and non-AA salts/reagents. (A3) Representative DSC curves fitted with non-two-state model. The real line is the overall curve obtained. Dashed lines shows separated unfolding peaks after deconvolution with T_m1, T_m2, and T_m3 indicated. (A4) Shifts of transition temperatures between formulations with and without excipients. (B1−4) The corresponding DSC profiles of JM2 formulations.

evidenced here with differential T_m shifting on JM1 and JM2 and with a previous report of strong destabilizing effect on C_H2 of the Fc fragment of an IgG1 antibody.[52]

In order to evaluate the effect of AAs/salts on the mAb conformational stability, we also obtained the FT-IR spectra of JM1 and JM2 in the mAb solution formulation with various additives (Figure 6). FT-IR spectra are useful for characterizing the composition of secondary structures in proteins and peptides. Amide I, as the most sensitive region, is generated from the C═O stretching vibration of the amide group coupled with in-phase bending of the N−H bond and stretching of the C−N bond.[53,54] Each frequency in the amide I region, detected between 1700 and 1600 cm^{-1}, corresponds to a particular secondary structure. The major peak around 1638 cm^{-1} corresponding to the dominant β-sheet structure of both mAbs did not shift upon addition of various excipients, suggesting that these excipients did not cause detectable changes in the secondary structure of the mAbs. The variation in the peak intensity is likely due to the concentration changes after repeated lyophilization−reconstitution cycles.[31] Also, the intensity variation near the 1600 cm^{-1} region was caused by the background signals from the 150 mM AAs/salts

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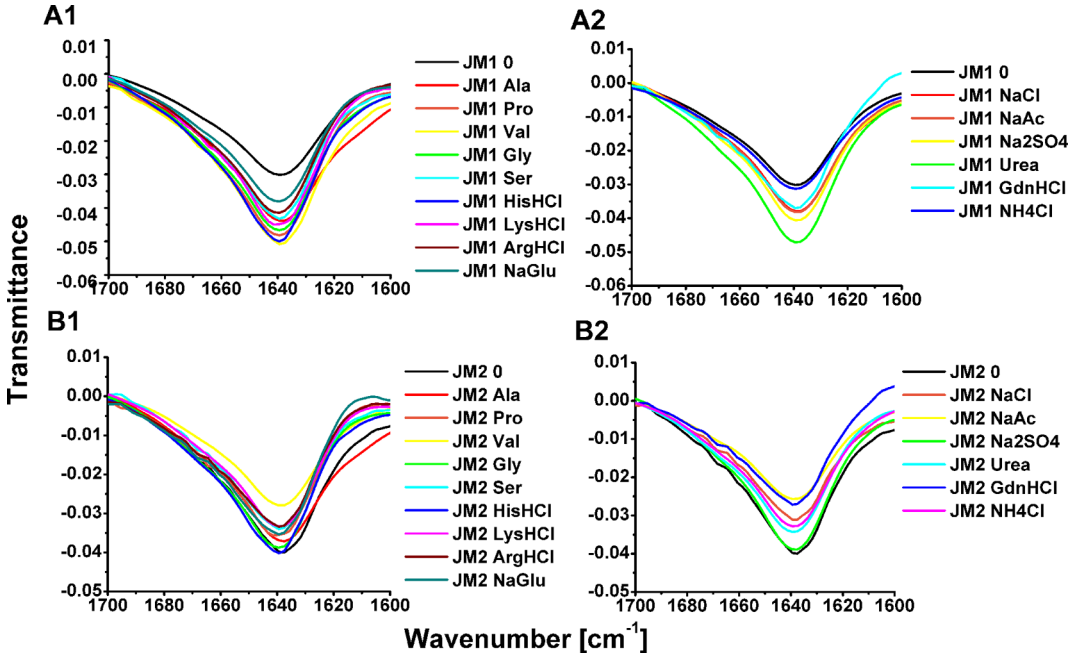

Figure 6. FT-IR spectra of amide I region of JM1 (A1 and A2) and JM2 (B1 and B2) in formulations with various excipients.

in the liquid formulation, although background subtraction has been performed.

CONCLUSION

Here we reported the impact of nine amino acids, four salts, and two chaotropic reagents on the viscosity and stability of two IgG1 antibody liquid formulations. We observed that AAs/salts, such as HisHCl, LysHCl, ArgHCl, NaGlu, NaCl, NaAc, Na_2SO_4, GdnHCl, and NH_4Cl, could significantly decrease the viscosity of JM1 and JM2 formulations in 20 mM HisHCl at pH 6.0. Such viscosity-lowering effects by these excipients do not cause detectable mAb aggregation, conformational changes, or decreased storage stability in the JM1 and JM2 formulations, except for GdnHCl, which induces 30−40% monomeric mAb loss after storage under 40 °C for 2 weeks. AAs/salts used in this study could be explored further for mAb formulation screening. With our further investigation of the relationship between the physiochemical properties of these excipients and the solution properties of the mAb formulations, we envision certain selected excipients could also serve as molecular probes to further reveal the molecular mechanism governing the protein−protein interaction, which could guide the future design of high-concentration mAb formulations.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.molpharmaceut.5b00643.

Chemical structure and solution viscosity of amino acids and representative salts; 3D excitation−emission-intensity fluorescence spectra of JM1 and JM2 in formulations with various 150 mM excipients; viscosity measurement of different BSA solutions with various viscometers (PDF)

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Notes

The authors declare no competing financial interest.

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