REPORT

Preferred interactions of trehalose, L-arginine.HCl and sodium chloride with therapeutically relevant IgG1 monoclonal antibodies

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ABSTRACT

Preferred interactions of weakly interacting formulation excipients govern their effect on the equilibrium and kinetics of several reactions of protein molecules in solution. Using vapor pressure osmometry, we characterized the preferred interactions of commonly used excipients trehalose, L-arginine.HCl and NaCl with three therapeutically-relevant, IgG1 monoclonal antibodies that have similar size and shape, but differ in their surface hydrophobicity and net charge. We further characterized the effect of these excipients on the reversible self-association, aggregation and viscosity behavior of these antibody molecules. We report that trehalose, L-arginine.HCl and NaCl are all excluded from the surface of the three IgG1 monoclonal antibodies, and that the exclusion behavior is linearly related to the excipient molality in the case of trehalose and NaCl, whereas a non-linear behavior is observed for L-arginine.HCl. Interestingly, we find that the magnitude of trehalose exclusion depends upon the nature of the protein surface. Such behavior is not observed in case of NaCl and L-arginine.HCl as they are excluded to the same extent from the surface of all three antibody molecules tested in this study. Analysis of data presented in this study provides further insight into the mechanisms governing excipient-mediated stabilization of mAb formulations.

Introduction

Monoclonal antibodies (mAbs) are a dominant class of therapeutic proteins that have been approved for the treatment of a variety of diseases in humans.1-4 Subcutaneous administration of mAb products increases their relative ease of use and enhance patient compliance.5 Such drugs typically require high concentration formulations due to the high dosage requirements of mAb therapies and the upper limit on injection volume for subcutaneous administration.6,7 A major challenge in the development of high concentration mAb formulations is the increased propensity for physical stability issues like aggregation and high viscosity.8,9 Apart from employing protein engineering approaches,10-12 physical stability of high concentration mAb solutions can also be enhanced through addition of various excipients like sugars, salts and amino acids.13,14 Due to the lack of clear mechanistic understanding of excipient-mediated stabilization, current approaches to formulation development involve high-throughput screening of several excipient combinations to identify a formulation that minimizes mAb instability.15,16

A rational approach to formulation development requires both a detailed understanding of reaction pathways responsible for aggregation and high viscosity of mAb formulations, as well as the impact of protein-excipient interactions on the kinetics and equilibrium of critical reactions within these pathways. Significant work has been undertaken to understand and identify the critical steps involved in mAb aggregation and high viscosity,17-20 but detailed understanding of the nature of mAb-excipient interaction is currently lacking.

Through their seminal papers, Schellman, Timasheff21-24 and others have laid the foundation for the preferential interaction theory framework that describes the nature and magnitude of protein-excipient interactions in terms of the preferential interaction co-efficient (Γ(μ3)). Using a modified Wyman-linkage function,25 one can estimate the effect of excipient addition on the free energy of protein reactions from the value of Γ(μ3). Thus, the magnitude of protein-excipient interactions can be linked quantitatively to the effect of excipient addition on the kinetics and equilibrium of protein reactions in aqueous solutions. Hong et al.26 have developed a vapor pressure osmometry based method to determine Γ(μ3). This method has the advantage of requiring smaller volume of protein solution, faster acquisition times and higher precision in the estimation of preferential interaction co-efficient, as compared to the traditional dialysis/densimetry method.

Although previous studies have explored the interaction of co-solutes with small model proteins,27-30 preferential interaction data for excipients generally regarded as safe with therapeutically relevant mAbs in a concentration range suitable for parenteral formulations is lacking. In this study, we characterized the nature and magnitude of preferential interactions of trehalose, L-arginine.HCl and sodium chloride (NaCl), with three therapeutically relevant IgG1 mAbs. We also characterized
the effect of these excipients on several physical instabilities of
the antibody molecules, including protein unfolding and revers-
ible self-association in dilute solution, and aggregation and vis-
cosity at high protein concentration. Taken together, these data
provide further insight into the mechanism of protein stabiliza-
tion by these commonly used excipients.

Results

Antibodies

To understand the nature and magnitude of preferential inter-
actions of formulation excipients, we chose three therapeuti-
cally-relevant IgG1 mAbs. To estimate the molecular weight of
the three antibodies, we conducted a multi-angle light scatter-
ing (MALS) analysis on the native state monomeric antibodies
fractionated using a size exclusion chromatography (SEC) col-
umn. As shown in Table 1, all three antibodies have similar
molecular weights of ~145–150 kDa. The spatial aggregation
propensity (SAP) score, which quantifies the exposed hydro-
phobic surface area of an antibody, was calculated for each
energy minimized and equilibrated antibody structure.10 As
listed in Table 1, the SAP score is highly similar for mAbA and
mAbB at ~580, but is significantly higher for mAbC at ~677
units, indicating that mAbC is much more hydrophobic com-
pared to mAbA and mAbB. The charge on each antibody was
determined by assigning the predicted charged state for each
side chain in the computational antibody models at a pH of 5.5
units and summing the charges for solvent-exposed amino
acids. The positive and negative charges are similar for mAbA
and mAbB and the net charge is positive. mAbC has fewer pos-
itive charges and a similar number of negative charges com-
pared to mAbA and mAbB, and consequently has a lower net
positive charge. Thus, the three antibodies have similar shape
and size, but different surface hydrophobicity and net charge.

Preferential interactions of trehalose, L-arginine.HCl
and NaCl

We first determined the partial molar volumes of the three exci-
pients and the three antibodies in the 25 mM Na-acetate buffer
at pH 5.5. Table 1 lists the partial molar volume data (\( \bar{V}_m \)) for
the three antibodies. The partial molar volumes for mAbB and
mAbC are quite similar and equal to about 103.6 L/mol. The par-
tial molar volume for mAbA is slightly higher at 104.6 L/mol. Sup-
plementary Table 1 lists the partial molar volume data for the
three excipients as a function of excipient molality. The partial
molar volume for trehalose shows a linear trend and decreases
with increasing molality. The partial molar volume for NaCl is
also linearly related to the molality, but shows an increase with
increasing molality. The partial molar volume for L-arginine.HCl
shows a non-linear behavior with respect to its molality. The par-
tial molar volume of L-arginine.HCl increases with increasing
molality in the concentration range of 0–1 M, but the rate of
increase is lower at higher molality.

Next, we quantified the chemical potential derivatives \( \mu_{23,4} \)
and preferential interaction co-efficients (\( \Gamma_{p,3} \)) for the three exci-
pients using the method of Hong et al.20 with a series of 16
binary and ternary solutions each for trehalose and NaCl and
24 binary and ternary solutions each for L-arginine.HCl. The
osmolality values of the non-ideal ternary solutions deviate
from their theoretical ideal solution value, and it is this dif-
ference between ideal and non-ideal solution osmolality that is
used to capture the preferential interaction of excipients with
the protein surface using the method of vapor pressure osmom-
etry. The vapor pressure osmometry experiments were con-
ducted at room temperature and atmospheric pressure
conditions. As shown in Fig. 1, all three excipients have positive
values for \( \mu_{23,4} \) and consequently negative values of \( \Gamma_{p,3} \) in
the concentration range of 0–0.4 molal. Thus, all three excipien-
ts are excluded from the surface of all three mAb molecules
characterized in this study.

As shown in Fig. 1A, the chemical potential derivative \( \mu_{23,4} \)
for trehalose is relatively constant in the concentration range of
0–0.4 molal with a value of roughly ~240 kJ/mol of protein per
molar of trehalose for mAbA, ~270 kJ/mol per molal for
mAbB and ~400 kJ/mol per molal for mAbC. The preferential
interaction co-efficient for trehalose is linear with respect to
excipient molality (Fig. 1D). This is consistent with previous
literature for smaller globular proteins with trehalose and other
sugars like sucrose that exhibit preferential exclusion behav-
ior.29,31 As shown in Table 2, the \( \Gamma_{p,3} \) for mAbA and mAbB are
very similar and have a value of ~ −12 at 0.1 M concentra-
tion of trehalose. By the definition of preferential interaction
co-efficient, this means that addition of every molecule of pro-
tein requires the net removal of ~12 molecules of trehalose to
maintain trehalose chemical potential at 100 mM concentration
of trehalose. As seen in Fig. 1D and Table 2, the degree of exclu-
sion of trehalose is significantly higher from the surface of
mAbC antibody compared to mAbA and mAbB. As the three
antibodies have similar shape and sizes, but significantly differ-
ent surface hydrophobicity and net charge (Table 1), we con-
clude that the degree of exclusion of trehalose depends
significantly on protein surface features. The potential for

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Table 1. Surface and molecular attributes of mAbA, mAbB and mAbC. Molecular weights of mAbA, mAbB and mAbC were determined by analyzing the monomer peak eluted from a size exclusion chromatography column using a miniDawn TRESOS MALS detector. Partial molar volume of the antibodies was determined by performing a densimetry assay as a function of antibody concentration in 25 mM Na-acetate buffer at pH 5.5 at 20 °C. Total point charges were determined by summing the predicted point charges of solvent accessible residues at pH 5.5 using a homology model of mAbA, mAbB and mAbC. The surface aggregation propensity (SAP) score which is a measure of the surface hydrophobicity of mAb molecules was calculated using molecular dynamics simulations.

| Antibody | Molecular weight (kDa) | Partial molar volume (\( \bar{V}_m \)) (L/mol) | Charge | Hydrophobicity SAP score |
|----------|------------------------|---------------------------------|--------|-----------------------|
| mAbA     | 150.3 ± 0.4            | 104.6 ± 0.003                   | +151   | 38                    | 577 ± 8 |
| mAbB     | 143.8 ± 0.1            | 103.6 ± 0.02                    | +157   | 41                    | 581 ± 4 |
| mAbC     | 144.9 ± 0.1            | 103.7 ± 0.01                    | +145   | 30                    | 677 ± 5 |
greater exclusion from a hydrophobic surface is supported by the observations of Hong et al.\textsuperscript{32} who found that trehalose is excluded to a greater extent from aliphatic carbon in solution. Further analysis using molecular dynamic simulations will provide greater insight into the effect of protein surface features on the exclusion of trehalose.

The chemical potential derivative $\mu_{23,4}$ for NaCl is also relatively constant with increasing NaCl molality in the range of 0–0.4 molal with a constant value of $\sim$350 kJ/mol of protein per molal of NaCl (Fig. 1B). The magnitude of $\Gamma_{\mu,3}$ for NaCl is linearly related to excipient molality. As seen from the Fig. 1E and Table 2, the degree of exclusion is lower than that for trehalose, throughout the concentration range tested in this study. The magnitude for the degree of exclusion is similar for the three antibodies tested, and is roughly equal to $\sim$8.0 molecules of NaCl per molecule of protein at 0.1 M concentration of NaCl. Thus, to maintain the chemical potential of NaCl, roughly 8 molecules of NaCl need to be removed for the addition of every protein molecule. The molarity of NaCl for the mAbB...
Table 2. Best fit equations for the preferential interaction co-efficient at constant co-solute chemical potential, $\Gamma_{23,4}$, and transfer free energy, $\Delta\mu_{2,\text{transfer}}$, as a function of excipient molality. The preferential interaction co-efficient data for trehalose and NaCl was fitted using a linear fitting algorithm and that for L-arginine.HCl was fitted using a non-linear fitting algorithm in Origin. The error bars in the bracket denote the standard error of fit. Transfer free energy values, $\Delta\mu_{2,\text{transfer}}$, were calculated by integrating the fitting equations for $\mu_{23,4}$ as a function of excipient molality ($m_3$) for the indicated combinations of protein and excipient at room temperature using vapor pressure osmometry technique.

| Excipient       | Protein | $\Gamma_{23,4}$ (mol/mol) | $\Delta\mu_{2,\text{transfer}}$ (kJ/mol) |
|-----------------|---------|---------------------------|------------------------------------------|
| Trehalose       | mAbA    | $-93.2 \pm 0.7$           | $257.2 m_2 - 33.0 m_2^2$                  |
|                 | mAbB    | $-106.8 \pm 0.8$          | $289.6 m_2 - 51.8 m_2^2$                  |
|                 | mAbC    | $-153.4 \pm 0.9$          | $429.3 m_2 - 96.1 m_2^2$                  |
| NaCl            | mAbA    | $-71.9 \pm 0.2$           | $332.1 m_2 - 10.9 m_2^2$                  |
|                 | mAbB    | $-77.6 \pm 0.3$           | $350.3 m_2 - 17.4 m_2^2$                  |
|                 | mAbC    | $-77.0 \pm 0.2$           | $340.0 m_2 - 19.0 m_2^2$                  |
| L-arginine.HCl  | mAbA    | $-32.2 \pm 1.1$           | $165.9 m_2 + 138.5 m_2^2 - 20.2 m_2^3$   |
|                 | mAbB    | $-24.4 \pm 0.9$           | $141.8 m_2 + 178.6 m_2^2 - 21.5 m_2^3$   |
|                 | mAbC    | $-53.5 \pm 1.2$           | $268.9 m_2 + 244.7 m_2^2 - 56.0 m_2^3$   |

Experiments was limited below 0.2 M as mAbB formed precipitates at NaCl concentration above 0.2 M.

For L-arginine.HCl, the chemical potential derivative $\mu_{23,4}$ shows a linear trend with increasing L-arginine.HCl concentration ranging in value between 150 to 450 kJ/mol per molal in the concentration range of 0-0.4 molal of L-arginine.HCl (Fig. 1C). The $\Gamma_{23,4}$ value shows a non-linear dependence on the excipient molality (Fig. 1F). The preferential interaction co-efficient decreases at a higher rate at higher concentrations of L-arginine.HCl. This behavior has previously been observed by Schneider et al. with smaller globular proteins like lysozyme, $\alpha$-chymotrypsinogen and bovine serum albumin (BSA). The magnitude of preferential interactions is very low at the low concentrations typically used for subcutaneous formulations. For example, at a concentration of ~0.1 M the value of $\Gamma_{23,4}$ is ~ −4 mol of L-arginine.HCl per mol of protein. Thus, addition of one molecule of protein requires the net removal of ~4 molecules of L-arginine.HCl to maintain the chemical potential of L-arginine.HCl in a solution containing L-arginine.HCl, buffer, water and mAb. The magnitude of preferential interaction co-efficient for L-arginine.HCl is similar in the case of three antibodies. Thus, at least within the experimental error, no significant difference was found for the interaction of L-arginine.HCl with antibody surface features like hydrophobicity and net charge.

The preferential interactions of weakly interacting excipients modulates the thermodynamic equilibrium of protein reactions in solution by differentially changing the chemical potential of the reactant and product protein configurations in the solution. The change in the chemical potential of a protein molecule in the presence of an excipient molecule compared to a condition in the absence of the given excipient molecule is called the transfer free energy, $\Delta\mu_{2,\text{transfer}}$. The transfer free energy for a given protein excipient combination can be calculated using the following equation.

$$\Delta\mu_{2,\text{transfer}} = \int \mu_{23,4} \, dm_3$$

Thus, knowing the chemical potential derivative as a function of excipient molality, one can determine the transfer free energy for each protein in a given excipient solution. Fig. 2 and Table 2 list the transfer free energy for the three antibody molecules as a function of excipient molality. As exclusion of the co-solvent molecules leads to an unfavorable interaction between the protein and the co-solute at constant co-solute molality, the transfer free energy for the native state protein is positive for all combinations of antibodies and excipients tested in this study.

The trend of transfer free energy was found to be linear with respect to co-solute molality for both trehalose and NaCl. The transfer free energy was found to be non-linear, with the slope of the curve increasing at higher concentrations of L-arginine.HCl (Fig. 2). For all three antibodies, the transfer free energy is lowest in the case of L-arginine.HCl and has a magnitude of roughly $\sim 18$ kJ/mol at an L-arginine.HCl concentration of ~0.1 molal (Table 2). The transfer free energy for trehalose and NaCl are similar in magnitude for mAbA (Fig. 2A) and mAbB (Fig. 2B) up to at least 0.2 molal concentration. Due to the enhanced exclusion of trehalose from the surface of mAbC (Fig. 2C), the transfer free energy for trehalose is significantly higher ($\sim 84$ kJ/mol at 0.2 molal trehalose) than that for NaCl ($\sim 70$ kJ/mol at 0.2 molal NaCl).

**Effect of excipient addition on mAb unfolding and reversible self-association**

To further understand the nature of excipient-protein interaction, we characterized the effect of excipient addition on two commonly reported parameters for protein formulations: shift in the midpoint transition temperature ($T_{m}$) in differential scanning calorimetry (DSC) analysis and dynamic light scattering (DLS) interaction parameter ($k_D$). To characterize the effect on unfolding, we carried out a DSC assay to identify the midpoint melting transition temperature ($T_{m}$) of the antibodies in the presence and absence of excipients. Supplementary Table 2 lists the $T_{m}$ values for the various domains of the three antibodies tested in this study. In all DSC runs, except in the presence of buffer alone and with trehalose for mAbA and in the presence of trehalose for mAbC, the antibody aggregated irreversibly by the end of the thermal scan. Thus, the transitions are irreversible and cannot be analyzed using assumptions of thermodynamic equilibrium. Nonetheless, in several protein formulation studies an increase in the $T_{m}$ values has been found to be correlated with an increase in the conformational stability of mAb molecules. Thus, we included the data on $T_{m}$ shifts for comparison to other literature. Fig. 3 indicates the deviation of $T_{m}$ values, $\Delta T_{m}$, for the various domains of the three antibodies tested in this study. As shown in Fig. 3, trehalose stabilizes the Fab and P2 transition of the mAbA antibody (Fig. 3A) and the F3 and P1...
transition of mAbB (Fig. 3B). In both cases, the stabilization is greater in the case of the Fab domain compared to either P1 or P2 transition. Although trehalose is excluded to a greater extent from the mAbC at room temperature, it has a destabilizing effect on the Tm values of Fab and P1 transition of the mAbC (Fig. 3C). This could be attributed to the dependence of \( G_m \) on temperature. The effect of L-arginine.HCl on antibody melting seems to depend upon the individual domain. L-arginine.HCl stabilizes the P1 and Fab domains of mAbA, and Fab domain of mAbC. It destabilizes the P2 transition of mAbA, P1 and Fab domain of mAbB and P1 and P2 transitions of mAbC. The effect of NaCl also seems to depend upon the antibody domain. NaCl stabilizes the P1 and P2 transitions of mAbA and the Fab domains of mAbB and mAbC, whereas it destabilizes the Fab domain of mAbA, P1 transition of mAbB and P1 and P2 transition of mAbC.

To characterize the effect of excipient addition on reversible self-association, we determined the DLS interaction parameter \( k_D \), as this parameter has been previously shown to be a good predictor of protein-protein interaction.\(^{34,35}\) In general, an increase in the \( k_D \) value indicates lowering of the protein-protein interaction. The DLS interaction parameter was characterized for the three mAbs in the presence of trehalose, L-arginine.HCl and NaCl, and in the presence of buffer alone.

**Figure 2.** Transfer free energy for mAbA (A), mAbB (B) and mAbC (C). Transfer free energies, \( \Delta \mu_{transfer} \), for mAbA, mAbB and mAbC were calculated for the transfer from 25 mM Na-acetate buffer at pH 5.5 to the same buffer containing, additionally either trehalose (black), sodium chloride (red) or L-arginine.HCl (blue) at 20°C.

**Figure 3.** \( \Delta T_m \) analysis for the Fab, P1 and P2 transitions of mAbA (A), mAbB (B) and mAbC (C). Shift in Tm values with respect to 25 mM Na-acetate buffer alone, \( \Delta T_m \), are plotted. Positive \( \Delta T_m \) values indicate an increase in the Tm value compared to that in the presence of buffer alone and vice versa. NaCl and L-arginine.HCl were used at a final concentration of 100 mM, whereas trehalose was used at a final concentration of 200 mM.
As shown in Fig. 4, colloidal stability decreases with the addition of trehalose in the case of mAbC, NaCl in the case of mAbA and mAbB and for L-arginine.HCl in the case of mAbA and mAbB. Interestingly, a decrease in protein-protein interaction is observed in the case of trehalose with mAbA and mAbB, and for NaCl and L-arginine.HCl with mAbC.

**Effect of excipient addition on the accelerated aggregation kinetics of high-concentration solutions of mAbs**

Weakly-interacting excipients can modulate the aggregation rate of proteins in solution by differentially changing the transfer free energy of the native state and the transition state for the rate determining step in the aggregation cascade. To probe the effect of excipient addition on their aggregation behavior, mAbA, mAbB and mAbC were incubated at an elevated temperature. The incubation temperature was chosen so as to obtain 5–10% aggregation in a period of 10–15 days. The low aggregation rate was purposefully chosen to obtain data that would be representative of the storage stability of mAb formulations where an aggregate content of more than 5% over the shelf life is undesirable and raises concerns of potential immunogenicity of protein aggregates. The incubated mAb solutions were fractionated on a size-exclusion chromatography column to determine % monomer left in solution as a function of time. To ensure that the loss in monomer was primarily due to aggregation and not due to fragmentation or any other process, we also tracked the area under the curve for the corresponding fragment and aggregate peaks. As shown in Supplementary Fig. 1, monomer peak was identified in the aggregated sample by using the MALS detector. The resulting data for monomer loss was fitted to a first-order rate law to determine the monomer loss rate constant. To contextualize the impact of excipient addition on the aggregation reaction energetics compared to the impact on the energetics of the entire native antibody molecule, we determined the change in activation barrier upon excipient addition for a hypothetical single step, rate determining reaction, \( \Delta \mu_{2, \text{transfer}} \) using the following equation:

\[
\Delta \mu_{2, \text{transfer}} \text{ TRANSITION STATE} = - RT \ln \left( \frac{k}{k_0} \right)
\]

Table 3 shows the aggregation kinetics of the three antibodies in the presence of trehalose, L-arginine.HCl and NaCl. The data is presented in the form of \( k/k_0 \) where \( k \) represents the first order monomer loss rate constant for the given excipient and \( k_0 \) represents the monomer loss rate constant for the given antibody molecule in the presence of 25 mM Na-acetate, pH 5.5 buffer alone. As can be seen from Table 3, significant stabilization/destabilization effects are observed in the case of mAbA. For mAbB and mAbC, trehalose has slight stabilization of \( \sim 1.4 \)-fold for the aggregation kinetics compared to buffer alone. This means that the transition state barrier is increased by \( \sim 0.8 \) kJ/mol compared to the native state. This is a very small increase compared to the transfer free energy of \( \sim 50 \) kJ/mol for mAbB and \( \sim 84 \) kJ/mol for mAbC native state. Trehalose also has a stabilizing effect of \( \sim 2 \)-fold on the aggregation kinetics of mAbA. The transition state barrier in this case is increased by 1.3 kJ/mol.

NaCl and L-arginine.HCl increase the aggregation kinetics of mAbA. The transition state barrier is decreased by about 2.1 kJ/mol for NaCl and by 1.3 kJ/mol for L-arginine.HCl. Given that the transfer free energy of the native state mAbA in NaCl and L-arginine.HCl is 33.0 kJ/mol and 18.0 kJ/mol, respectively, the observed decrease is again a small fraction of the observed change in the chemical potential for the entire antibody surface.

**Figure 4.** \( k_0 \) analysis for mAbA (A), mAbB (B) and mAbC (C) in 25 mM Na-acetate, pH 5.5 buffer alone or in combination with 100 mM sodium chloride, 100 mM L-arginine.HCl or 200 mM trehalose. DLS measurements were conducted at 25°C to estimate the apparent diffusion coefficient of mAbA and mAbB in the concentration range of 0–25 mg/mL and from 0–4 mg/mL for mAbC. The resulting data were fit with Origin's linear fitting algorithm to estimate the DLS interaction parameter \( k_0 \) value, which has been previously shown to correlate with the reversible self-association of proteins in solution. Error bars represent the standard error in the estimation of the slope of the linear fit.
Effect of excipient addition on the dynamic viscosity of high-concentration solutions of mAbs

The viscosity of high concentration mAb solutions has previously been shown to be dependent upon a combination of long- and short-range interactions between the protein molecules that result in the formation of protein networks.38-40 Excipients are added to mAb solutions to minimize these interactions and break the interaction network, thereby reducing viscosity for a given mAb at a particular concentration and temperature.41,42 To determine the effect of excipient addition on the viscosity of high concentration mAb solutions, we characterized protein solutions using cone and plate rheometry at a protein concentration of 100 mg/mL and a temperature of 20°C.

As shown in Fig. 5, mAbA solution in buffer alone has a low viscosity of 4.1 cP. Addition of trehalose, NaCl and L-arginine.HCl does not result in any significant changes in the viscosity of mAbA solutions. For mAbB, the viscosity in the presence of buffer alone is considerable higher and is equal to 24.6 cP. Addition of 100 mM NaCl results in the reduction of viscosity to 20 cP. In the presence of 100 mM L-arginine.HCl, the solution viscosity of mAbB is halved and it reduces to a value of 12.1 cP. On the other hand, addition of 200 mM trehalose results in an increase in the viscosity of mAbB solution. The reduction in viscosity observed in the presence of NaCl and L-arginine.HCl hints at the contribution of charged and aromatic side-chains to protein-protein interaction in mAbB solutions at high concentrations. mAbC solution also has a high viscosity of 34.9 cP in the presence of buffer alone. Interestingly, addition of 100 mM NaCl, 100 mM L-arginine.HCl or 200 mM trehalose, reduce viscosity of mAbC solutions to 7.6, 4.8 and 8.2 cP, respectively.

Discussion

Physical stability of high concentration liquid mAb formulations is governed by several reversible reactions of the protein in solution that can be classified into either conformational (protein unfolding) or colloidal (protein-protein self-association) destabilization pathways. Conformational destabilization results in an increase in the protein-water interfacial area
whereas colloidal destabilization results in a decrease in the interfacial area. The addition of excipients to mAb solutions affects the conformational and colloidal destabilization pathways through their preferential interactions with the protein surface. Excipients that are excluded from the surface of mAb molecules increase the chemical potential of the protein-water interface. Excipients that are preferentially bound to the mAb surface decrease the chemical potential of the protein-water interfacial area. Thus, addition of excluded co-solutes favors a reduction in the protein-water interface. Addition of preferentially bound co-solutes, on the other hand, favors an increase in protein-water interfacial area. Thus, preferentially excluded excipients promote conformational stabilization and colloidal destabilization. Preferentially bound excipients favor conformational destabilization and colloidal stabilization.

In this study, we characterized the preferential interactions of three commonly used excipients, trehalose, NaCl and L-arginine-HCl, with the surface of three mAbs that have similar size, shape and charge, but differ in their surface hydrophobicity as represented by the SAP hydrophobicity score and net charge. We also characterized the effect of excipient addition on the reversible self-association, aggregation and viscosity behavior of high concentration solutions of these three antibodies. The knowledge of excipient preferential interaction with the native state of the mAb molecule, along with its effect on the reversible self-association, aggregation and viscosity behavior, provides an insight into the mechanisms governing the effect of the various excipients on the physical stability of mAb molecules.

Trehalose competes with water molecules to interact with the protein surface through its ability to form hydrogen bonds with amino acids on the surface of the mAb molecules. In this study, we find that trehalose is excluded from the surface of the antibody molecule at all concentrations of trehalose in the range of 0–0.4 molal. The degree of exclusion is linearly related to the concentration of trehalose molecule (Fig. 1D and Table 2). This exclusion is in agreement with previous studies that observed preferential exclusion of sugars and polyols from the surface of protein molecules.19,20 Xie et al.29 have characterized the preferential interaction of trehalose with RNaseA, a small globular protein. The authors find that trehalose is excluded from the surface of the protein molecule, yielding a preferential interaction co-efficient of −4.33 mol/mol at 0.5 M concentration. This value is lower than that observed for the three mAb molecules (~−60 mol/mol for mAbA and mAbB and ~−87 mol/mol for mAbC at 0.5 M trehalose) in our study. The lower magnitude of $\Gamma$ is expected as preferential interaction co-efficient is an additive function that increases with increasing protein-water interfacial area. Thus, RNaseA (13.7 kDa), which is a much smaller protein compared to the mAb molecules (~150 kDa), would have a smaller protein-water interfacial area, and consequently a lower magnitude of preferential interaction co-efficient. Interestingly, in this study, we found that trehalose is excluded to a significantly greater extent from the surface of mAbC molecule compared to mAbA and mAbB (Fig. 1A, D). This suggests that trehalose exclusion is strongly dependent on the nature of the protein surface. This result is supported by the findings of Hong et al.22 who found that, for model small molecule compounds in solution, the exclusion of trehalose from aliphatic carbon is greater compared to other surfaces. Molecular level details of the interaction of trehalose molecule with amino acid side chains are beyond the scope of this study.

NaCl competes with water molecules through its electrostatic interactions with the protein surface. L-arginine-HCl can similarly interact with the protein surface through electrostatic interactions. In addition to the electrostatic interactions, L-arginine-HCl can also form cation-pi interactions with the aromatic side chains on the protein surface.43 In this study, we find that NaCl is excluded from the surface of the three antibody molecules at all concentrations in the range of 0–0.4 molal (Fig. 1E, Table 2). The degree of exclusion is also linearly related to the concentration of NaCl. At lower concentrations, L-arginine-HCl is neither excluded nor bound to the mAb surface (Fig. 1F, Table 2). As the concentration increases, L-arginine-HCl is strongly excluded from the surface of the mAb molecules. The degree of exclusion follows a non-linear trend with increasing concentrations of L-arginine-HCl. The preferential exclusion observed for NaCl from the surface of mAb molecules is consistent with the results obtained by Arakawa et al.44 for the interaction of 1 M NaCl with the surface of small globular proteins like lysozyme (~5.8 mol/mol at pH 7.0 in 1 M NaCl), BSA (~16.5 mol/mol at pH 7.0 in 1 M NaCl) and $\beta$-lactoglobulin (~3.97 mol/mol at pH 7.0 in 1 M NaCl). The degree of exclusion for the smaller proteins is lower than that for mAb molecules (~−80 mol/mol at 1 M NaCl) in our study. As expected, the magnitude of preferential exclusion is directly related to the protein size and follows the order mAb (~150kDa) > BSA (~66.5 kDa) > lysozyme (~14.3 kDa) ~ $\beta$-lactoglobulin (~18.4 kDa). Interestingly, our data suggests that the exclusion trend is observed even at the lower concentrations of NaCl that are used for parenteral formulations. This observation of preferential interaction at low salt concentrations supports the specific ion effect observed for the charge screening ability of various salts by Gokarn et al.45 The non-linear trend in the exclusion behavior of L-arginine-HCl was also previously observed by Schneider et al.27 for three small globular proteins lysozyme, $\alpha$-chymotrypsinogen and BSA. As in our study, Schneider et al.27 observed a strong exclusion from the protein surface at high L-arginine-HCl concentration for lysozyme (~5.0 mol/mol at 1 M L-arginine-HCl), $\alpha$-chymotrypsinogen (~9.6 mol/mol at 1 M L-arginine-HCl) and BSA (~21.5 mol/mol at 1 M L-arginine-HCl). These values are much lower than that obtained for the mAbs in our study (~−205.0 mol/mol at 1 M L-arginine-HCl). At low concentrations, L-arginine-HCl interacted favorably with the surface of lysozyme (~0.44 mol/mol at 0.1 M L-arginine-HCl) but, as in this study with mAb molecules, was neither excluded nor bound to the surface of BSA (~0.06 mol/mol at 0.1 M L-arginine-HCl) and $\alpha$-chymotrypsinogen (~0.015 mol/mol at 1 M L-arginine-HCl). The magnitude of preferential interaction of L-arginine-HCl at high concentration also increases with protein size and follows the order mAb > BSA > $\alpha$-chymotrypsinogen > lysozyme. Due to the non-linear trend in its preferential interactions, L-arginine-HCl interacts more favorably than NaCl with the protein surface at low concentrations, but more unfavorably at higher concentration. The favorable interaction at low concentration could be due to the ability of L-arginine to interact with aromatic residues in addition to electrostatic interactions. In a
molecular dynamics (MD) simulation study, Shukla et al. demonstrated the formation of reversible L-arginine clusters at high concentrations. These clusters were shown to be excluded strongly from the surface of the protein molecule. Thus, L-arginine.HCl is excluded strongly from the protein surface at high concentrations.

The preferential interaction data provides an insight into the potential conformational stabilization behavior of these excipient molecules through the preferential interaction mechanism. For an excipient to affect the conformational stability of the mAb molecules, it must be excluded to a greater extent from the surface of a partially unfolded molecule compared to the native state of the molecule. Protein unfolding involves an increase in the protein-water interfacial area. As the magnitude of preferential interactions scales with increasing surface area, preferentially excluded excipients are excluded to a greater extent from the surface of the partially unfolded protein molecule compared to the native state. This results in stabilization of the protein unfolding pathway due to the addition of excluded excipients. Trehalose and NaCl are both excluded from the surface of the antibody. Thus, trehalose and NaCl are expected to enhance the conformational stability of mAb molecules. L-arginine.HCl on the other hand is not excluded from the surface of the antibody molecules at low concentrations (< 0.2 M) typically used in parenteral formulation. Therefore, it is not expected to have any effect on the conformational stabilization behavior of mAb molecules.

Excipients modulate the colloidal stability of protein solutions by modifying the electrostatic and hydrophobic self-interactions of the protein molecule. The net protein self-interactions can be determined using the DLS interaction parameter, \( k_D \). The addition of salts to protein solutions reduces the electrostatic interactions between protein molecules through the charge screening effect. At low concentrations, addition of salts reduces the colloidal stability of mAb molecules that have repulsive electrostatic interactions and enhances the colloidal stability of mAb molecules with attractive electrostatic interactions. As attractive protein-protein interactions result in a decrease in the protein-water interfacial area, addition of preferentially excluded excipients to protein solutions is expected to increase the protein-protein interaction reactions and reduce colloidal stability of the protein solution. In this study, addition of the salts NaCl and L-arginine.HCl results in a decrease in the colloidal stability of mAbA and mAbB, whereas there is an increase in the colloidal stability of mAbC solution (Fig. 4A-C). This indicates that the underlying interactions of the mAbA and mAbB molecules at pH 5.5 are electrostatically repulsive in nature and that for mAbC are electrostatically attractive in nature. Although, L-arginine.HCl reduces the colloidal stability of mAbA and mAbB, the destabilization is much lower compared to that due to the addition of NaCl. This might be due to the fact that, at the low concentration of 100mM, L-arginine.HCl is not preferentially excluded from the surface of the mAbA and mAbB molecules (Fig. 1C, F). Thus, it does not reduce colloidal stability through the preferential exclusion mechanism. NaCl on the other hand is excluded from the protein surface and destabilizes the protein surface through the preferential exclusion mechanism. Similarly, mAbC is also colloidal stabilized by L-arginine.HCl to a much greater extent compared to NaCl, presumably due to the lack of destabilization through the preferential exclusion mechanism. Trehalose is a non-electrolyte and is therefore not expected to affect electrostatic interactions through the charge shielding mechanism. As it is preferentially excluded from the protein surface, it is also expected to promote protein-protein interactions. This is indeed the case with mAbC where addition of trehalose results in a significant decline in the \( k_D \) value (Fig. 4C). Surprisingly, addition of trehalose resulted in an increase in the colloidal stability of mAbA and mAbB (Fig. 4A, B). MD simulation studies might provide further insight into the molecular mechanism behind this observed effect.

Excipients can affect the aggregation kinetics of mAb formulations through their effect on the conformational and colloidal stability pathways of the mAb molecules. As trehalose and NaCl are excluded from the surface of all three mAb molecules, they are expected to enhance the conformational stability of the mAb molecules. L-arginine.HCl, which is neither excluded nor bound to the mAb surface at low concentrations (< 0.2 M), is expected to have no effect on the conformational stability of the mAb molecules. From the data on colloidal stabilization, we find that trehalose enhances the colloidal stability of mAbA and mAbB and decreases the stability of mAbC. L-arginine.HCl and NaCl reduce the colloidal stability of mAbA and mAbB and enhance the colloidal stability of mAbC. From the aggregation kinetics data, it is clear that trehalose is able to minimize the aggregation kinetics of mAbA and mAbB molecules through conformational and colloidal stabilization. Although, trehalose reduces the colloidal stability of mAbC, it reduces the aggregation kinetics in this case as well. The addition of L-arginine.HCl has no effect on the aggregation kinetics of mAbB and mAbC (Table 3), presumably due to the lack of conformational stabilization. The addition of L-arginine.HCl enhances the aggregation kinetics of mAbA possibly due to the reduction in mAbA colloidal stability through a charge screening effect (Table 3). NaCl similarly increases the aggregation kinetics for mAbA through colloidal destabilization due to charge screening. The addition of NaCl to mAbB does not have a clear effect on the aggregation kinetics of the molecule. Addition of NaCl enhances both the conformational and colloidal stability of the mAbC molecule (Table 3), which results in reduced rates of mAbC aggregation upon addition of NaCl. The diverse effects of excipient addition on the aggregation kinetics of the three mAb molecules is indicative of the complexity of the underlying mechanism of protein aggregation. Nonetheless, excipients that enhance both conformational and colloidal stability are found to reduce aggregation rates.

The viscosity of high concentration mAb formulations has been shown to be much higher than that for single domain globular proteins. This has been attributed to the ability of the multi-domain antibodies to form long range cross-linked networks. Thus, reduction in mAb viscosity upon excipient addition is related to the ability of the excipient to break down this network. It has been previously shown that electrostatic interaction between the Fc and Fab regions of the mAb molecules results in a network formation that is the underlying cause for high viscosity observed in high concentration mAb formulations. Thus, addition of electrolytes like NaCl and L-arginine.HCl results in a decrease in the viscosity of these mAb formulations. L-arginine.HCl is exceptionally good at...
reducing the viscosity of high concentration mAb formulations (Fig. 5A-C). This might be attributed to the ability of L-arginine.HCl to crowd out interactions between the F\textsubscript{ab} and F\textsubscript{c} domains and to the lack of preferential exclusion-driven enhancement of protein-protein interaction. Addition of trehalose results in an increase in the viscosity of mAbB solutions. This might be attributed to the preferential exclusion of trehalose from the mAbB surface, which results in the promotion of burial of protein surface and potentially the additional exposure of charged groups. Interestingly, addition of trehalose to mAbB solutions results in a decrease in viscosity (Fig. 5C). The $k_D$ data indicates a decrease in mAbC colloidal stability upon trehalose addition (Fig. 4C). Thus, the decrease in mAbC viscosity upon trehalose addition (Fig. 5C) suggests that addition of trehalose impedes the ability of mAbC molecules to form a long-range, viscosity enhancing network.

In conclusion, we characterized the preferential interactions of commonly used formulation excipients trehalose, L-arginine.HCl and NaCl with three therapeutically relevant IgG1 mAbs. We find that trehalose, NaCl and L-arginine.HCl are excluded from the surface of all three antibody molecules at all excipient concentrations in the range of 0–0.4 molal. The magnitude of exclusion increases linearly with excipient molality for trehalose and NaCl and is non-linear for L-arginine.HCl. Interestingly, we also find evidence to suggest that the magnitude of trehalose exclusion depends on the nature of the protein surface. Taken together, the data presented here provides an insight into the conformational and colloidal stabilization behavior of these excipient molecules and their effect on the aggregation and viscosity of high concentration mAb formulations.

**Materials and methods**

**Molecular simulations**

Primary sequence data for the three antibodies was obtained from MedImmune. The crystal structures for the F\textsubscript{ab} fragment of mAbA (PDB code: 4ypg) and of mAbC (PDB code: 5anm) were obtained from the Protein Data Bank.\(^5^9\) The primary sequence of mAbB differed from that of mAbA only in the F\textsubscript{v} region. A homology model of the F\textsubscript{v} region of mAbB was generated using the RosettaAntibody protocol,\(^5^0\) then aligned with the mAbA structure to produce the full mAbB F\textsubscript{ab} structure. The three full antibody structures were generated according to the procedure outlined by Brandt et al.\(^5^1\) using a computational model of an IgG1 antibody as a template\(^5^2\) and assuming a G\textsubscript{o} glycosylation pattern.

All simulations employed the CHARMM22\(^5^3\) force field parameters for proteins, the TIP3P model for water,\(^5^4\) and the CHARMM36 parameters for carbohydrates. Simulations were performed in NAMD\(^5^5\) in water boxes extending a minimum of 13 Å from the antibody surface and periodic boundary conditions. All simulations were performed in the NpT ensemble at 1 atm and 298 K. Simulations were performed at pH 5.5, as set based on the protonation states of ionizable residues. The N terminals were protonated and the C terminals deprotonated, with parameters taken from the CTER and NTER parameters in CHARMM. Arginine and lysine residues were protonated and aspartic acid and glutamic acid residues were deprotonated. Because the pKa of histidine is close to the simulation pH, the protonation states of histidine residues were set using the PROPKA framework,\(^5^6\) which takes the pH and the local hydrogen bonding opportunities into account. Chloride counter-ions were placed randomly within the simulation box so that the system was "charge neutral". Explicit water molecules were randomly placed within the simulation box. The systems were initially energy minimized, then equilibrated for about 10 ns, with the final 2 ns used for SAP calculations.\(^1^0\)

**Materials**

mAbA, mAbB and mAbC were provided in proprietary liquid formulations by MedImmune. High purity trehalose (Catalog: T9449), L-arginine.HCl (Catalog: 11039), NaCl (Catalog: S7653) and glacial acetic acid (Catalog: A6283) were purchased from Sigma. Buffers were prepared by dissolving the required reagents in 18.2 MΩ water and adjusting pH using either 5 N sodium hydroxide or 1 N hydrochloric acid. The resulting solutions were then diluted to the required final volume using a volumetric flask, filtered through a 0.22 μm filter and used within one week of preparation. Protein samples were dialyzed overnight against 25 mM sodium acetate, pH 5.5 buffer using Slide-A-Lyzer MINI dialysis devices (Thermo Fisher Scientific) with three changes of dialysis buffer. Protein concentrations were determined using the absorbance at 280 nm wavelength and extinction co-efficients for the three antibodies provided by MedImmune. To achieve the final target concentration, protein samples were either diluted with the dialysis buffer or concentrated using an Amicon spin concentrator (EMD Millipore). Samples were stored at 4 °C to minimize aggregation and other degradation reactions.

**Vapor pressure osmometry**

Vapor pressure osmometry is a relatively quick and easy technique that can be used to determine the osmolality of aqueous solutions using only 10 μL of sample solution. Hong et al.\(^2^6\) have described the use of vapor pressure osmometry measurements to estimate the co-solute preferential interaction co-efficient at constant co-solute chemical potential, $\Gamma_{\mu_3}$ in a 4-component solution containing water, protein, co-solute and a buffer. In this experimental setup, two series of solutions with varying concentrations of excipient are prepared: a binary series containing only the buffer and excipient and a ternary series containing the excipient, buffer and protein. The two series are then assayed using a Wescor Vapro 5600 vapor pressure osmometer (Elitech Group) to determine osmolality of solution with known excipient molality. The resulting data is then used to determine the preferential interaction co-efficient using the difference in the osmolality of the binary and ternary solutions after the osmolality contributions of buffer alone and buffer
in the presence of protein have been taken into account.

\[ \Gamma_{\mu_3} = - \frac{\mu_{23,4}}{\mu_{33,4}} \]

\( \Gamma_{\mu_3} \) — Excipient preferential interaction co-efficient at constant excipient chemical potential and constant temperature and pressure in mol/mol where,

\[ \mu_{23,4} = \frac{RTD_{23,4}Osm}{m_2m_3} \]

Where,

\[ \Delta_{23,4}Osm = \{Osm(m_2, m_3, m_4) - Osm(m_2, m_4)\} - \{Osm(m_3, m_4) - Osm(m_4)\} \]

\[ Osm(m_2, m_3, m_4) \] — Fitting equation for the osmolality vs m3 value for ternary solution.

\[ Osm(m_2, m_4) \] — Intercept of the fitting equation for the osmolality vs m3 value for ternary solution. This value accounts for the osmolality contribution of buffer and protein in the absence of co-solute.

\[ Osm(m_3, m_4) \] — Fitting equation for the osmolality vs m3 value for binary solution.

\[ Osm(m_4) \] — Intercept of the fitting equation for the osmolality vs m3 value for binary solution. This value accounts for the osmolality contribution of buffer alone in the absence of co-solute.

\[ m_3 \] — Excipient molality in mol/kg

\[ m_2 \] — Protein molality in mol/kg.

The term \( \mu_{33,4} \) is determined by taking the partial derivative of the binary solution osmolality with respect to the excipient molality at constant temperature and pressure. Thus,

\[ \mu_{33,4} = \left( \frac{RT}{m_3} \right) \left( \frac{\partial Osm(m_3, m_4)}{\partial m_3} \right)_{T,P} \]

In this study, 25 mM sodium acetate at pH 5.5 was used as the buffer. To ensure high accuracy in the determination of excipient concentration, all solutions were prepared gravimetrically using a high-precision (instrumental error ~0.1 mg) Mettler Toledo weighing balance. Stock solutions of NaCl, L-arginine.HCl and trehalose were prepared at a concentration of ~1 molal in 25 mM Na-acetate buffer at pH 5.5. The stock solutions were then serially diluted using 25 mM sodium acetate, pH 5.5 buffer to obtain a set of 16 stock solutions in the case of trehalose and NaCl and 24 solutions in the case of L-arginine.HCl. It was found that this large number of dilutions was required to minimize the standard error in the estimation of fitting parameters for the osmolality versus molality curves for both binary as well as ternary solutions. To obtain binary solutions, roughly 40 \( \mu \)L of the excipient stock dilution was mixed with about 40 \( \mu \)L of buffer. To obtain ternary solutions, roughly 40 \( \mu \)L of the stock excipient dilution was mixed with ~40 \( \mu \)L of concentrated antibody solution (> 100 mg/mL). The weight of each aliquot was determined using the Mettler Toledo balance to get an accurate estimate of excipient molality in each dilution. The sample tubes, thus prepared, were wrapped in Parafilm to minimize evaporation losses during storage at 4 °C. All samples were analyzed using the Wescor VAPRO 5560 osmometer (Elitech group) within five days of preparation.

Sealed sample tubes were brought to room temperature and centrifuged for 10 mins at 14,000 g before analyzing them on the osmometer. Sample analysis on the osmometer was carried out following manufacturer’s protocol. Although the VPO instrument is very precise, it has a tendency to drift away from the calibration values over time. To minimize this drift and enhance measurement accuracy, the instrument was recalibrated after every four measurements until the osmolality values for the manufacturer-provided standards were stabilized. Also, to minimize errors from evaporation, osmolality standards were discarded two hours after opening of the vial.

To determine the partial molar volume of the excipients and proteins, solutions were prepared gravimetrically containing varying concentrations of the excipients (8 solutions from 0 to ~1 M) or protein of interest (6 dilutions from 0 to 25 mg/mL). Next, densities of these solutions were measured using a high-precision Anton Paar 4500 densimeter. The density data thus obtained was then used to determine the partial molar volume using the following equation:

\[ \bar{V}_3 = \frac{1}{\rho_3} + (1 - z_3) \left( \frac{\partial V}{\partial z} \right)_{T,P,z_3 = 0} \]

**Differential scanning calorimetry**

Thermal melting scans for mAbA, mAbB and mAbC were determined using a MicroCal VP-DSC microcalorimeter. Protein samples were dialyzed overnight with three exchanges of the desired dialysis buffer. The dialysate from the last exchange was used as the reference buffer for the microcalorimeter. All samples and reference buffers were degassed prior to manual loading into the sample and reference chambers of the microcalorimeter. Protein samples were analyzed at a concentration of ~1 mg/mL, in the temperature range of 25–90 °C and at a scanning rate of 1 °C/min.

**Accelerated aggregation assay**

The \( T_{onset} \) temperature was defined as the lowest temperature at which the normalized heat capacity of the first transition peak in a DSC thermogram reaches 5% of its peak value. To ensure an accelerated aggregation condition that would be representative of aggregation under storage conditions, antibodies were incubated at a temperature lower than the \( T_{onset} \) temperature. Within this limit, the temperature was set such that a percent
aggregates, and % aggregate for each sample. The resulting data was recorded in terms of the area under the curve for the monomer and aggregate peaks. The resulting data was then analyzed using the Chemstation software to determine the concentration of mAb from small oligomers and high molecular weight aggregates. After loading 20 μL of this sample onto the column. Day 0 was set within the 800 s–1 range. Viscosity data was collected using three independent samples to account for instrumental and sample loading errors.

**Dynamic light scattering and kD analysis**

A DynaPro Nanostar (Wyatt Technology) instrument was used to record the diffusion coefficient. To determine the DLS interaction parameters for various formulations, 4–5 dilutions of mAbA and mAbB were prepared in the concentration range of 0–25 mg/mL. mAbC was found to have a high degree of reversibility association at higher concentration. To ensure monodisperse protein solutions, mAbs dilutions were limited to 4–5 concentrations in the range of 0–4 mg/mL. To minimize scattering due to suspended foreign particle, protein samples were centrifuged at 14,000 g for 1 hour before analysis using DLS. Only samples with % polydispersity values lower than 15% were used in the determination of kD values. Based on the apparent diffusivity values (Dm), the kD value was determined using the following equation:

\[ D_m = D_0(1 + k_DC) \]

**Viscosity measurement using cone and plate rheometry**

The dynamic viscosities of mAbA, mAbB, and mAbC formulations at 100 mg/mL concentrations were determined using DHR-3 (TA Instruments) cone and plate rheometer. A 1 degree cone with 20 mm diameter was used to limit the sample volume to ~80 μL per measurement. The protein samples were centrifuged for 10 mins at 14,000 g to remove suspended particles. Care was taken to avoid bubble formation between the cone and plate surfaces. To minimize sample evaporation during measurement, the temperature of plate was set at 20 °C and handling time was minimized. To eliminate low and high shear rate errors, dynamic viscosity was determined for shear rates set within the 800–2000 s⁻¹ range. Viscosity data was collected using three independent samples to account for instrumental and sample loading errors.

**Disclosure of potential conflict of interest**

No potential conflicts of interest were disclosed.

**Acknowledgements**

We would like to thank MedImmune for supporting this study and providing the antibody molecules. We would also like to thank Dr. Curt Schneider and Dr. Fabienne Courtois for their helpful discussions on experimental procedures for vapor pressure osmometry and accelerated aggregation kinetics, respectively.

**Funding agency**

This work was supported by MedImmune.

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