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Research Paper

Effect of Ions on Agitation- and Temperature-Induced Aggregation Reactions of Antibodies

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Purpose. The impact of ions on protein aggregation remains poorly understood. We explored the role of ionic strength and ion identity on the temperature- and agitation-induced aggregation of antibodies.
Methods. Stability studies were used to determine the influence of monovalent Hofmeister anions and cations on aggregation propensity of three IgG_2 mAbs. The C_H2 domain melting temperature (T_{m1}) and reduced valence (z^*) of the mAbs were measured.
Results. Agitation led to increased solution turbidity, consistent with the formation of insoluble aggregates, while soluble aggregates were formed during high temperature storage. The degree of aggregation increased with anion size ($F^- < Cl^- < Br^- < I^- < SCN^- \sim ClO_4^-$) and correlated with a decrease in T_{m1} and z^*. The aggregation propensity induced by the anions increased with the chaotropic nature of anion. The cation identity (Li^+, Na^+, K^+, Rb^+, or Cs^+) had no effect on T_{m1}, z^* or aggregation upon agitation.
Conclusions. The results indicate that anion binding mediates aggregation by lowering mAb conformational stability and reduced valence. Our observations support an agitation-induced particulation model in which anions enhance the partitioning and unfolding of mAbs at the air/water interface. Aggregation predominantly occurs at this interface; refreshing of the surface during agitation releases the insoluble aggregates into bulk solution.

KEY WORDS: aggregation; Hofmeister effect; ions; monoclonal antibody; reduced valence.

INTRODUCTION

Protein aggregation phenomena present significant challenges in the production and stability of protein therapeutics. Over-expressed proteins are often unable to fold, forming instead insoluble aggregates. Obtaining the correct fold may require modification of the protein sequence (1), combination of the therapeutic protein with a carrier protein (2), or refolding (3). Even if the native fold is achieved, proteins in pharmaceutical formulations can form soluble and insoluble aggregates under environmental stresses (4–6). There is concern that both aggregate types can enhance the immunogenic response in patients (7). In addition, sufficiently large, insoluble aggregates may act as particles, which have been reported to mechanically block capillaries and cause reduced microcirculation in postischemic patients (8). Ionic excipients in protein formulations, utilized as buffering agents, viscosity modifiers, or tonicity agents, have an impact on protein aggregation. The degree of impact varies according to their ionic strength and identity.

Ions can be ranked according to their ability to modulate both protein solubility and conformational stability in terms of the Hofmeister series (9). At moderate to high salt concentrations (>300 mM), kosmotropic ions decrease protein solubility and increase conformational stability, while chaotropic ions have the opposite effect. At molar concentrations, salts have been employed in protein denaturation (10,11), crystallization (12), and modulating protein/protein interactions (13). The source of Hofmeister effects remains in dispute, however there is increasing recognition that ions interact directly with proteins, rather than affecting protein solubility and stability through changes in bulk water structure (14).

The effects observed at high salt concentrations are not necessarily applicable in the sub-molar salt regime where charge neutralization can play a critical role in governing protein solubility as well as stability. Even low concentrations of kosmotropic ions help solubilize proteins, screening charged protein side chains and making protein/protein interactions less energetically favorable (15). In a series of papers (16–18), Collins has defined the solution interactions between proteins and salts in terms of the relative affinity of ions for the molecular dipole of water. The model is based on

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the analysis of Hofmeister ions using gel sieving chromatography (19) and is most readily applicable to monovalent ions, for which there are no confounding avidity effects. Briefly, in aqueous solution, inner sphere ion pairs are hypothesized to preferentially form by ions of opposite sign and similar size. Ions with low charge density (chaotropes) interact poorly with water and, by pairing with each other, allow for an increased number of water/water interactions. In contrast, ions with high charge density (kosmotropes) are able to form more favorable electrostatic interactions with other small ions than with water; the distance between the opposite charges is shorter in the anion/cation pairs than in the ion/water pairs. This theoretical description provides a good basis for discussion of the interaction of dissolved salts with charged protein side chains, particularly in the low concentration salt regime commonly used in protein formulations.

While many protein therapeutics have been developed as low-concentration dosage forms (20), monoclonal antibodies (mAbs) are often desired to be delivered in high concentrations (>50 mg/mL) *via* subcutaneous injection, posing solubility, stability, and viscosity challenges. Buffering salts are added to maintain formulation pH, while non-buffering salts may be added to achieve isotonicity or reduce solution viscosity (21,22). Additionally, salts are employed during production and the carry-over of ions from chromatographic purification steps can potentially impact protein stability in the final dosage form. Though frequently used in production processes and marketed formulations, the effects of ions in low concentrations on protein stability remain poorly understood. In previously published work (23), our lab showed that the formation of soluble mAb aggregates increased with the ionic strength of buffer ions. In this report, we explore the role of both ionic strength and ion identity on temperature- and agitation-induced aggregation. We probed the influence of ions on mAb aggregation using three IgG2 mAbs, mAb1, mAb2, and mAb3 (Tables I and II), and a series of monovalent Hofmeister anions (F^-, Cl^-, Br^-, I^-, PC^-, SCN^-) and cations (Li^+, Na^+, K^+, Rb^+, Cs^+). The ions are a subset of those studied by Collins and their interactions with proteins have been discussed extensively in the literature. By studying these ions, rather than only those ions employed in pharmaceutical development, we aim to understand the underlying mechanisms governing ion-induced mAb aggregation.

MATERIALS AND METHODS

Monoclonal Antibodies

Bulk drug lots of the IgG2 monoclonal antibodies mAb1, mAb2, and mAb3 were received from the Amgen Process Development Group at concentrations of 70 mg/mL. The primary structure of the constant domains (C_H1–C_H3 and C_L) was identical for the three mAbs. Select biophysical properties and the amino acid composition of the molecules appear in Tables I and II, respectively. The presented MW values and pI values were approximated based on the amino acid composition using ExPasy (24).

Table I. Biophysical Properties of the Studied Antibodies

	Isotype	MW (kD)	pI	pI HC	pI LC
mAb1	IgG2, kappa LC	145	8.2	7.6	8.6
mAb2	IgG2, kappa LC	144	7.8	8.0	7.1
mAb3	IgG2, kappa LC	144	8.3	8.6	6.2

Table II. Amino Acid Composition of the Studied Antibodies

	mAb1	mAb2	mAb3
A	34	34	36
C	18	18	18
D	25	24	25
E	35	36	33
F	26	25	24
G	47	46	40
H	10	12	10
I	13	12	11
K	39	39	40
L	48	49	43
M	7	6	8
N	23	23	24
P	47	46	45
Q	31	31	35
R	25	23	23
S	84	87	81
T	54	51	58
V	60	60	63
W	11	11	12
Y	25	26	27
Total	662	659	656

Ultrafiltration/Diafiltration

A Millipore (Billerica, MA) Labscale TFF system was used to prepare all mAb stock solutions in 10 mM acetate at pH 5.0 with or without 5% sorbitol. Prior to diafiltration all permeate lines were replaced and preconditioned with 2 L of water followed by a liter of 2 mM acetate buffer pH 5.0. During the diafiltration process, the buffer components in the protein solution were removed and exchanged with 2 mM acetate buffer pH 5.0. Three 30 K regenerated cellulose membranes (Millipore Pellicon XL) were used in the process. The steps were carried out at room temperature with a transmembrane pressure of 15 psi and a feed pressure of 30 psi. A total of 8 diavolumes of buffer were exchanged and further concentration was performed where needed to obtain a final concentration of 70 mg/mL. The pH was titrated to pH 5.0 with 0.05 N HCl. All molecules were analyzed by size-exclusion HPLC (SE-HPLC) pre and post UF/DF to check for potential aggregation during the process. The final acetate concentration was determined by HPLC.

Acetate Concentration Determination

The acetate concentration was measured using an HPLC method. Samples with <70 mg/mL protein were diluted 1:1 with 0.1 M phosphoric acid pH 2.00 to a final volume of 200 μL and centrifuged in a Millipore Microcon centrifugal filter (regenerated cellulose, 30,000 MWCO) for 10 min at

12,000 rpm. The filtrate was run on an Agilent 1200 HPLC equipped with a Supelco C-18 column (15 cm length×4.6 mm id, 3 μm) at a 1.0 mL/min flow rate for 7 min. Data was analyzed using the Chromeleon software package (Dionex, Sunnyvale, CA). Acetate concentration determined by comparing the area of the acetate peak to a standard curve derived from multiple injections of samples with known acetate concentrations.

Agitation Studies

Insoluble aggregate formation, leading to solution turbidity, was accelerated using a VWR (West Chester, PA) analog orbital shaker (model OS-500) operating at a speed of 500 rpm for 64.5 h at room temperature. Four Nalgene (Rochester, NY) polycarbonate boxes (133×133×51 mm) were placed in a square and secured in the center of the shaker platform with Velcro® and reinforced with tape. Each box contained a foam insert intended to hold 64 1.5 mL microcentrifuge tubes. The insert was modified to hold 16, evenly spaced 3 cc vials. The four boxes (64 vials) were run on the shaker simultaneously, allowing 16 formulations with a sample size of four to be tested in a single run.

Differential Scanning Calorimetry (DSC)

The DSC experiments were run on a VP-Capillary DSC system from Microcal (Northampton, MA) equipped with tantalum 61 cells, each having an active volume of 125 μL. Protein samples at 70 mg/mL were diluted to 1.5 mg/mL. The samples were scanned from 20°C to 90°C at a rate of 1.5°C/min with a 15 min equilibration at 20°C. A filtering period of 16 s was used and data was analyzed using Origin7.0 software (OriginLab Corporation, Northampton, MA). Thermograms were corrected by subtraction of control (buffer-only) scans. The corrected thermograms were normalized for protein concentration. The apparent melting temperatures (T_m) were obtained using the "Pick Peak" function of the software. The values reported are single experiments, with the exception of the mAb1 data reported in Fig. 6. The average and standard deviation of three melts is shown.

Quiescent Stability Study

Stability studies were performed in a buffer that consisted of 10 mM acetate pH 5 with 50 mM salts (Cl^-, Br^-, I^-, PC^-, SCN^-). The protein was sterile filtered (0.22 μm cellulose acetate filters), filled in 3 cc vials and put at 4°C and 45°C. At each time point, samples were visually inspected, the solution pH was measured, and aliquots were analyzed by SE-HPLC.

Turbidity Measurements (A360 nm and A400 nm)

Absorbance readings at 360 nm and 400 nm were made using an Agilent (Santa Clara, CA) 8,453 UV/Vis spectrophotometer equipped with UV/Vis Chemstation software. Immediately after the 64.5-h agitation period, samples were diluted 1:10 in water (10 μL of protein solution: 90 μL of water). A 70 μL quartz cuvette with a cell length of 1 cm was used to measure turbidity. Additional dilutions were made, as necessary, to keep the total absorbance below 1. The reported turbidity values are corrected for the dilution, thus a value of 7.8 is the result of measuring an absorbance of 0.78 after a 1:10 dilution of an agitation sample.

Size Exclusion Chromatography (SE-HPLC)

An Agilent 1200 was used to monitor aggregation using SE-HPLC. Samples were separated on tandem TSKG3000swxl column with the eluent being monitored by UV absorbance at 235 nm. The purity was assessed by determining the relative percent peak area of the aggregate, monomer and post peak regions using the Dionex (Sunnyvale, CA) Chromeleon software package.

During SE-HPLC analysis at each time point, a frozen mAb reference standard was injected multiple times (ten times for mAb1 and mAb2, four times for mAb3). The standard deviation in the % monomer of the reference standard was used to represent the error in the % monomer value for all samples at that time point.

Reduced Valence Measurements

Reduced valence (z^*) measurements were made using a combination of sedimentation velocity and electrophoretic mobility measurements (25). The z^* of a protein can be described using the relationship in Eq. 1, where μ is the electrophoretic mobility, k_B is Boltzmann's constant, T is the absolute temperature, D_t is the translational diffusion constant, and e is the elementary charge.

$$z* = \frac{\mu k_B T}{D_t e} \tag{1}$$

The term D_t can be defined in terms of the sedimentation coefficient (s), universal gas constant (R), molecular weight (M_w), partial specific volume (vbar), and solvent density (ρ) (Eq. 2).

$$D_t = \frac{sRT}{M_w(1-\bar{v}\rho)} \tag{2}$$

The vbar value for each of the mAbs was calculated using the primary structure and Sednterp v1.1 (26). The values obtained for the three mAbs differed by less than 0.0006 mL/g; the average value of 0.728 mL/g was used. Sedimentation coefficients were obtained using a Beckman Coulter (Fullerton, CA) XLI centrifuge. Samples containing 0.25 mg/mL protein were spun at 40,000 rpm and sedimentation was monitored using 280 nm pseudo-absorbance scans. Two-sector sedimentation velocity centerpieces were used with samples loaded into the "reference" and "sample" sides. Samples were equilibrated to 20°C before initiation of the experiments. The resulting data were analyzed using SedFit (27) to obtain the sedimentation coefficient. Solution densities were obtained in the absence of protein using an Anton Paar GmbH (Graz, Austria) DMA 5000 densitometer.

The electrophoretic mobility was determined using a Beckman Coulter PA 800 instrument and a 60 cm eCap amide capillary. Protein samples were prepared at 1 mg/mL and were injected immediately after the injection of an electroosmotic flow (EOF) marker. For most solutions,

0.02% (*v*/*v*) DMSO was used as the EOF marker with detection at 214 nm. Solutions containing iodide or thiocyanate included an injection of 2% (*v*/*v*) benzyl alcohol as the EOF marker and detection at 280 nm. The experiments were executed at 20°C with an applied potential of 12,000 volts. The value of μ was determined using the relationship in Eq. 3, in which t_{EOF} and t_{mAb} are the elution times of the EOF marker and mAb, C_t is the total capillary length, C_d is the capillary length before the detector (the distance traversed by the marker/protein prior to detection), and P is the applied potential.

$$\mu = \left(\frac{C_d}{t_{EOF}} - \frac{C_d}{t_{mAb}}\right)\left(\frac{C_t}{P}\right) \quad (3)$$

Samples were prepared by exchanging bulk mAb solutions (~70 mg/mL) into 10 mM acetate at pH 5.0 using a NAP5 desalting column (GE Healthcare). The resulting solutions (~32 mg/mL mAb) were spiked into appropriate salt solutions to obtain the desired formulation.

The calculated z^* values are based on single measurements of solution density and the sedimentation coefficient, in combination with two or more measurements of electrophoretic mobility. Variation in the last value translated to an error in z^* that did not exceed 0.01.

RESULTS

Agitation Studies

Agitation of 70 mg/mL mAb solutions for a period of 65 h resulted in increased solution turbidity. If stored statically, the solutions became clear, with a layer of protein aggregate at the bottom, consistent with the formation of insoluble aggregate particles. The degree of aggregation could be modulated by both the concentration and identity of ions present in the formulation. The studies were conducted using pH 5.0 formulations containing 2 mM acetate; the mAbs were able to provide adequate buffer capacity in this pH and protein concentration regime (23). The tested salts (50 mM of NaF, NaCl, NaBr, NaI, $NaClO_4$, NaSCN, KCl, RbCl, or CsCl) dissociated into monovalent anions and cations and are common components of the Hofmeister series. The solution turbidity at 360 nm was used as a semi-quantitative measure of aggregate formation. While this method cannot characterize the particles formed and faces limitations in cases where particle size varies significantly, it continues to be used for the comparison of particle-containing protein solutions (6) and was sufficient for ranking the tested solutions (Fig. 1, Panels A and B). Turbidity increased according to anion identity in the order $F^- < Cl^- < Br^- < I^- < PC^- < SCN^-$. Of the three mAbs, mAb3 appeared to be the most sensitive to salts: the mAb consistently displayed the highest turbidity values. The differential ability of fluoride, chloride and bromide to influence turbidity was most pronounced for mAb3, while differences between perchlorate and thiocyanate were clearest for mAb1 and mAb2. For all three mAbs, the control formulation, containing 2 mM acetate and no additional salt, was the least turbid post agitation. Agitation did not appear to cause a significant degree of soluble aggregate formation. Analysis of the mAb1 anion-series samples by size-exclusion HPLC (SE-HPLC) post agitation showed no significant differences in the % soluble aggregate, either within the series or in comparison to a non-stressed standard (Fig. 1, Panel C). The identity of the cation did not appear to influence aggregation; the turbidity post agitation was comparable across the studied cation series.

Turbidity increased with ionic strength. Solutions of 70 mg/mL mAb1 with 2 mM acetate (pH 5.0) containing 0 to 150 mM NaCl, NaI, NaSCN, LiCl, or CsCl were agitated for 65 h, after which the solution turbidity was measured (Fig. 2). The salt identity dependent effects observed at 50 mM were also seen over the extended concentration range. Turbidity was dependent on anion identity, increasing in the order NaCl < NaI < NaSCN. The turbidity values for the LiCl, NaCl, and CsCl solutions, however, were nearly identical at a given concentration, consistent with the cation identity not influencing insoluble aggregate formation.

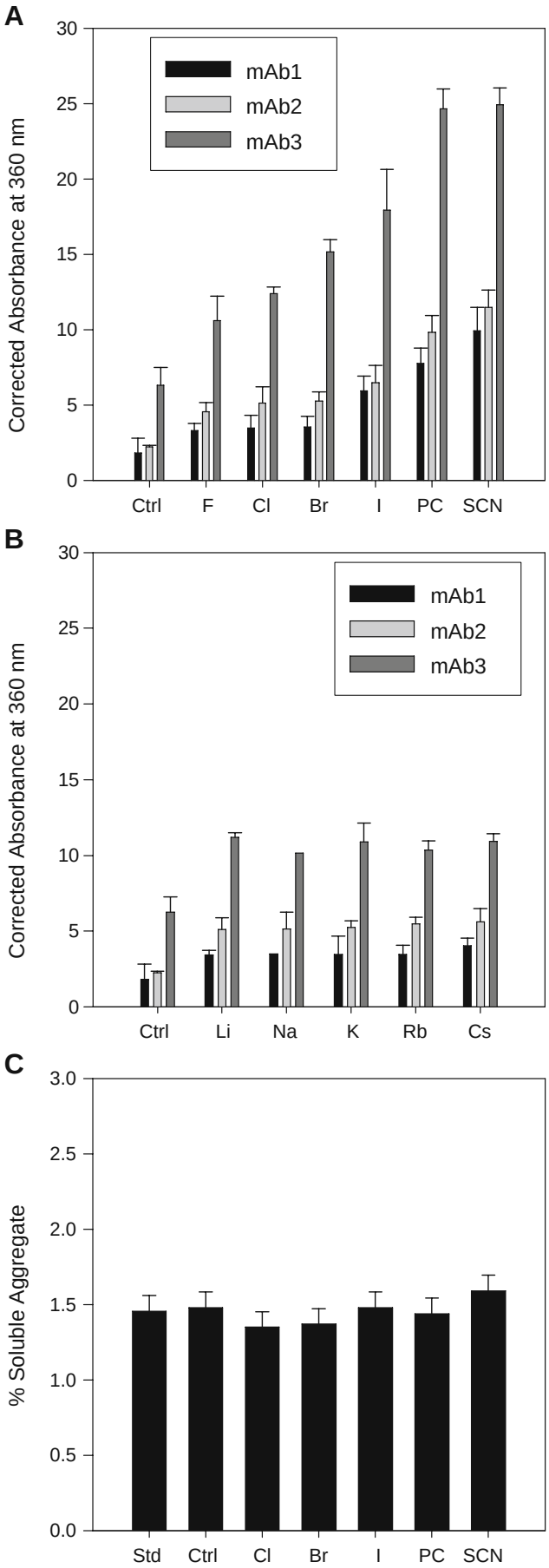
Quiescent Storage Stability Studies

The agitation studies showed a clear trend for the anions studied. An accelerated quiescent stability study at 45°C was used to determine if this trend was maintained in the absence of agitation. For comparison with the high-temperature stability study, mAb1 was stored for 26 weeks at 4°C. The three mAbs were formulated at 10 mg/mL with 10 mM acetate and a 50 mM salt at pH 5.0. A control containing 10 mg/mL mAb, 10 mM acetate, but no salt, was included. Soluble aggregate formation was monitored by SE-HPLC and proved to be the primary degradation pathway for the mAbs: the area lost by the main SE-HPLC peak was gained by a high molecular weight peak (Fig. 3). Stability was quantified in terms of the relative % main peak area (or % monomer, Fig. 4).

Of the three mAbs, mAb3 was the least stable during storage at 45°C. Aggregation was pronounced (Fig. 4, Panel C): the % monomer of the perchlorate- and thiocyanate-containing formulations fell below 30% (21.6% and 27.9%) after 4 weeks of storage. Both formulations were hazy and contained discrete visible particles (observable against a black background) in addition to soluble aggregates. The formulations containing halide ions had % monomer values after 4 weeks of 72.1%, 62.6%, and 38.0% for chloride, bromide, and iodide, respectively. At initiation of the study, the average % monomer of all the formulations was 99.3% with a standard deviation of 0.0%.

The ion identity trends observed for mAb3 were similar to those obtained for mAb1 and mAb2. The latter two mAbs did differ from mAb3 in two respects: (1) essentially no difference was seen between chloride and bromide and (2)

Fig. 1. Post-agitation absorbance at 360 nm as a function of anion (Panel A) or cation (Panel B) species, corrected for a 1:10 dilution. Solutions containing 70 mg/mL protein, 2 mM acetate, and 50 mM of a sodium or chloride salt were agitated for 65 h, after which the turbidity of the solutions at 360 nm was measured. The control solution contained no additional salt. Analysis of the anion samples by SE-HPLC post agitation showed no change in the amount of soluble aggregate *versus* a non-stressed standard (Panel C).



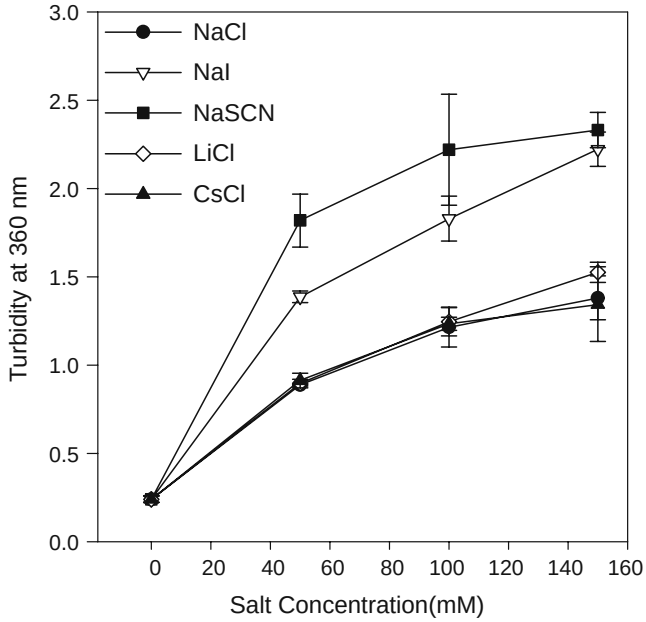


Fig. 2. Post-agitation absorbance at 360 nm as a function of salt concentration, corrected for a 1:10 dilution. Solutions containing 70 mg/mL mAb1, 2 mM acetate, and varying concentrations of a salt were agitated for 64.5 h, after which the turbidity of the solutions at 360 nm was measured.

thiocyanate was more destabilizing than perchlorate. At 45°C, the overall stability of the three mAbs could be ranked as mAb2>mAb1>>mAb3. From a comparison of the 4°C and 45°C data for mAb1, it was clear that the aggregation was accelerated at high temperatures (Figs. 3 and 4, Panel D). After 26 weeks of storage, no growth in soluble aggregate was observed for the samples stored at 4°C.

T_{m1} Measurements

The mAb melting temperature, derived from differential scanning calorimetry, was used as a measure of conformational stability (28). Data for mAb1 with varying levels of NaCl showed three melting transitions, apparent T_{m1-3} (Fig. 5), and were assigned to the C_H2, Fab, and C_H3 domains of the mAb based on previous work (29–31). The lowest melting temperature, that of the C_H2 domain, was most sensitive to salt concentration. Increasing the NaCl concentration from 0 to 150 mM resulted in a ~5°C decrease in T_{m1}. The traces obtained for mAb2 and mAb3 were analyzed similarly (data not shown), however the transitions for the F_{ab} and C_H3 overlapped and could not be independently assigned.

For all three mAbs, the apparent T_{m1} was sensitive to anion identity. This was studied using pH 5.0 solutions that contained 1.5 mg/mL mAb, 10 mM acetate, and 50 mM of salt. A control sample which did not contain the 50 mM salt was also tested. The impact of the anions on T_{m1} was $F^- < Cl^- < Br^- \sim I^- < PC^- \sim SCN^-$ (Fig. 6) and was greater for mAb1 than mAb2 or mAb3. The mAb T_{m1} was not sensitive to the identity of the cation; the value was decreased similarly by all of the chloride salts.

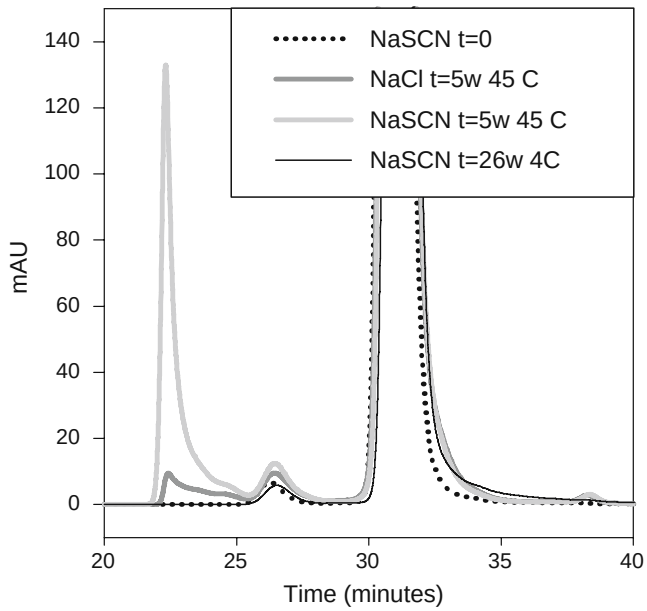


Fig. 3. Monomeric mAb1 elutes after 32 min by SE-HPLC. Aggregates elute earlier. A small amount of soluble aggregate can be observed in all of the time-zero chromatograms; the data for NaSCN (*dotted*) is shown. The NaCl (*dark grey*) and NaSCN (*light grey*) samples show significant aggregate growth after 5 weeks at 45°C. No aggregate growth was observed at 4°C after 26 weeks (NaSCN, *black*).

As in the agitation studies, the effect of ion concentration was tested using mAb1. The apparent T_{m1} value decreased as the concentration of the tested salt (NaCl, NaI, NaSCN, LiCl, or CsCl) was increased (Fig. 7). No cation-specific effect on T_{m1} was found: the T_{m1} values for mAb1 in NaCl, LiCl, and CsCl were in good agreement at all concentrations. A clear anion-specific effect was, however, evident and the impact of the anions could be ordered as $Cl^- < I^- < SCN^-$.

mAb Reduced Valence

The three mAbs studied were basic, with pI values of ~ 8. At pH 5.0, in low ionic strength solutions, the mAbs had a reduced valence (z^*) >8. The z^* was sensitive to the ionic strength of the solution, decreasing with salt addition and the three mAbs displayed similar z^* *vs.* salt concentration profiles (Fig. 8). As with the conformational stability, z^* was sensitive to anion, but not cation, identity (Fig. 9). In pH 5.0 solutions containing 10 mM acetate and 50 mM salt, the impact of the anions on z^* could be ranked as $F^- < Cl^- < Br^- \sim PC^- < I^- < SCN^-$.

DISCUSSION

Ion Binding Mediates Aggregation

The propensity for both agitation- and heat-induced aggregation increased as the charge density of the anion decreased; formulations containing thiocyanate were more prone to aggregation (soluble and insoluble) than those containing chloride. This observation was opposite to the trend for protein solubility expected from ions in the Hofmeister series, in which chaotropic (*salting in*) ions increase solubility. The increased aggregation propensity correlated with a decrease in the reduced valence of the protein and the apparent T_{m1}. The results can be rationalized using Collins' approach to the interaction of ions and proteins. The ions employed in our studies differ in their ability to bind to water; the kosmotropes (F^-, Li^+) bind tightly to water and the chaotropes (e.g. PC^-, SCN^-, Rb^+, Cs^+) bind weakly (16). Even though the ions in both the cation and anion series range in their ability to bind water, it was the anions that had the larger impact on the mAbs. The cations, independent of their charge density, did not influence aggregation, z^*, or T_{m1}.

The connection between anion binding and the subsequent reduction in the mAb z^* is straightforward; association of a negatively charged ion with a positively charged protein decreases the reduced valence of the protein. A reduction in z^*, either due to anion identity or concentration, correlated with increased aggregation and solution turbidity after agitation. This is consistent with studies in which a reduction in net charge has been linked to increased aggregation in the form of amyloid fibril formation (32–34).

The dependence of the measured T_{m1} on anion, but not cation, identity is consistent with previously reported data. The T_m of ribonuclease A was reduced most significantly by SCN^-, followed by Br^- and Cl^- (11). Ahmad *et al.* showed that NaCl, KCl, RbCl, and CsCl, in the presence of GdnHCl, were equally destabilizing to protein structure (35). The authors also noted that LiCl was somewhat destabilizing relative to the other alkali-metal salts, however our results do not show this. The observed destabilization was significantly less than that induced by either bromide or perchlorate salts. The cause of the destabilization has been previously described as the result of *salting in* the backbone amides, thus favoring the unfolded state in which more of the amides are exposed (9). Salt addition is not always destabilizing; addition of salts to the protein ubiquitin increased, rather than decreased, conformational stability (36)

The biophysical measures employed herein were predictive of mAb aggregation propensity as a function of added ions; however, their utility for comparing different mAbs under a given solution condition requires further study. Despite the significant differences in aggregation propensities between the three mAbs, as observed in both the agitation and accelerated quiescent studies, the proteins could not be readily differentiated by their T_{m1} or z^* values. In most cases, the T_{m1} values obtained for mAb3, the mAb with the highest aggregation propensity, are similar to or intermediate between those obtained for mAb1 and mAb2; the same observation can be made with respect to the recorded z^* values. It is possible that the different levels of aggregation could be the result of the carryover of ions or other impurities, however given the diafiltration procedure employed, this seems unlikely.

We attempted to correlate the amino acid sequence of the mAbs to the differences in aggregation propensity. The primary structure of the three mAbs was 88% sequence identical with all of the differences appearing in the variable regions (V_H and V_L); the sequence identity in these regions was 73%. The hydrophobicity of the molecules was compared based on their grand average hydropathicity (GRAVY) index (37) scores. Given the degree of sequence identity, it is not

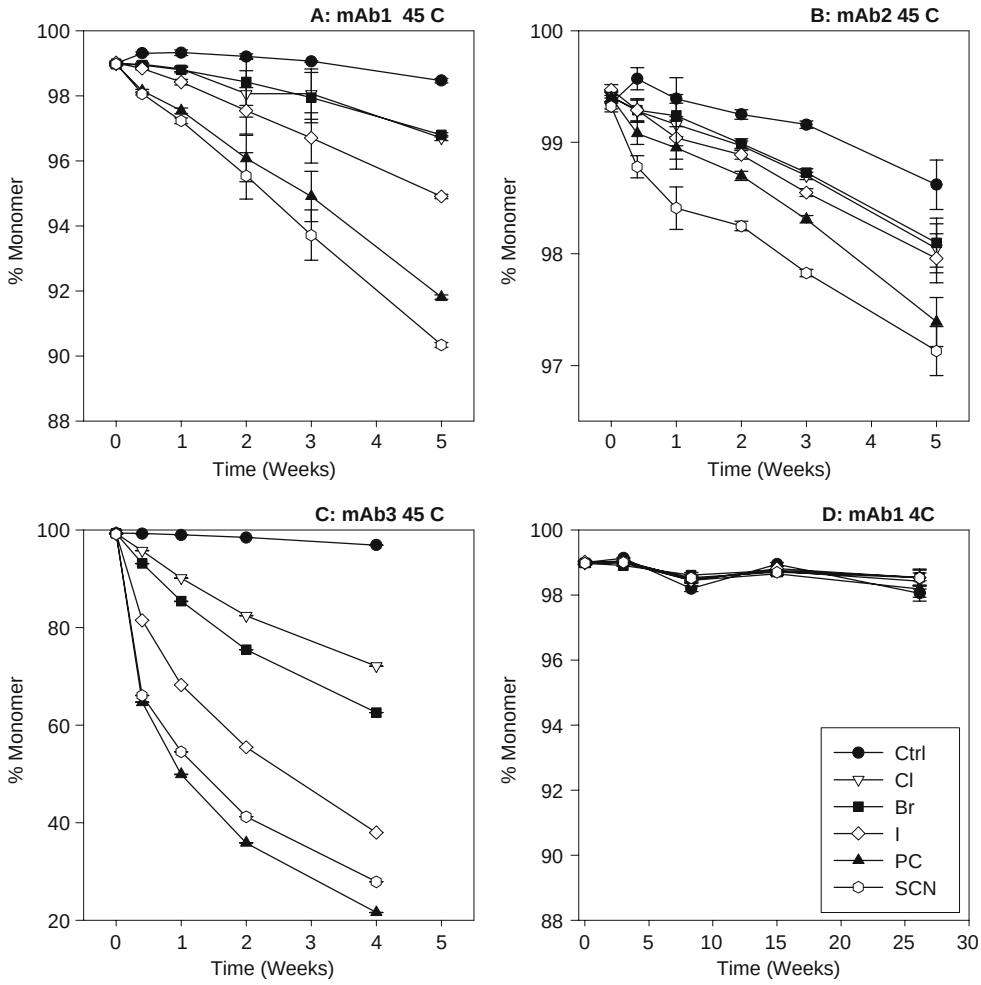


Fig. 4. Quiescent, 45°C SE-HPLC stability data for mAb1, mAb2, and mAb3, in Panels A, B, and C, respectively. Stability data for mAb1 at 4°C appears in Panel D. Extended storage at the high-temperature condition results in the conversion of the monomeric mAbs to soluble aggregates. The *error bars* are based on repeated injection of a reference standard (see Materials and Methods).

surprising that the three mAbs score similarly: −0.37, −0.40, and −0.37, for mAb1, mAb2, and mAb3 respectively. The CDR sequences within the variable domains displayed only 38.5% sequence identity. The GRAVY scores in these regions were −0.28, −0.86, −0.36, for mAb1, mAb2, and mAb3 respectively; the value for mAb3 was intermediate between that of mAb1 and mAb2.

Heat- *Versus* Agitation-induced Aggregate Formation

The solutions in the accelerated quiescent studies formed significant amounts of soluble aggregates, reflected in the loss of monomer by SE-HPLC (Fig. 3). After 5 weeks of storage at 45°C, however, the solutions of mAb1 and mAb2 remained transparent and free of visible particles. The mAb3 solutions, with the exception of the control, also remained transparent and contained only a few visible particles. The aggregation products formed under agitation stress were strikingly different, though the effect of the ions on the aggregation propensity remained qualitatively consistent. Agitation resulted in turbid solutions, ranging from hazy to opaque (Fig. 1 Panels A and B). The solutions of mAb1 were analyzed by size exclusion chromatography post-agitation and no significant changes in soluble aggregate *vs*. the control solution were found. The lack of soluble aggregate may either be due to particles that were formed post-agitation, effectively scavenged soluble aggregates present in solution, or it also may be indicative of the two aggregate types forming through different mechanisms. It is reasonable to assume that at high-temperature and quiescent storage, soluble aggregates form as the result of the collision of partially unfolded molecules. The visible particles observed for mAb3 may have been the result these particles growing to an aggregate size which was no longer soluble or they may have resulted from a separate mechanism.

Insoluble aggregate formation occurred considerably faster with agitation at room temperature than with quiescent storage at 45°C. This is consistent with a model in which insoluble aggregates form at the air/water interface rather than in bulk solution. With agitation, the air/water interface is dramatically expanded and, unlike in quiescent storage, is continuously refreshed. The hydrophobic surface can support the formation of large aggregates with low aqueous solubility, resulting from proteins partitioning to the surface, unfolding, and interacting. Under static storage conditions, such aggre-

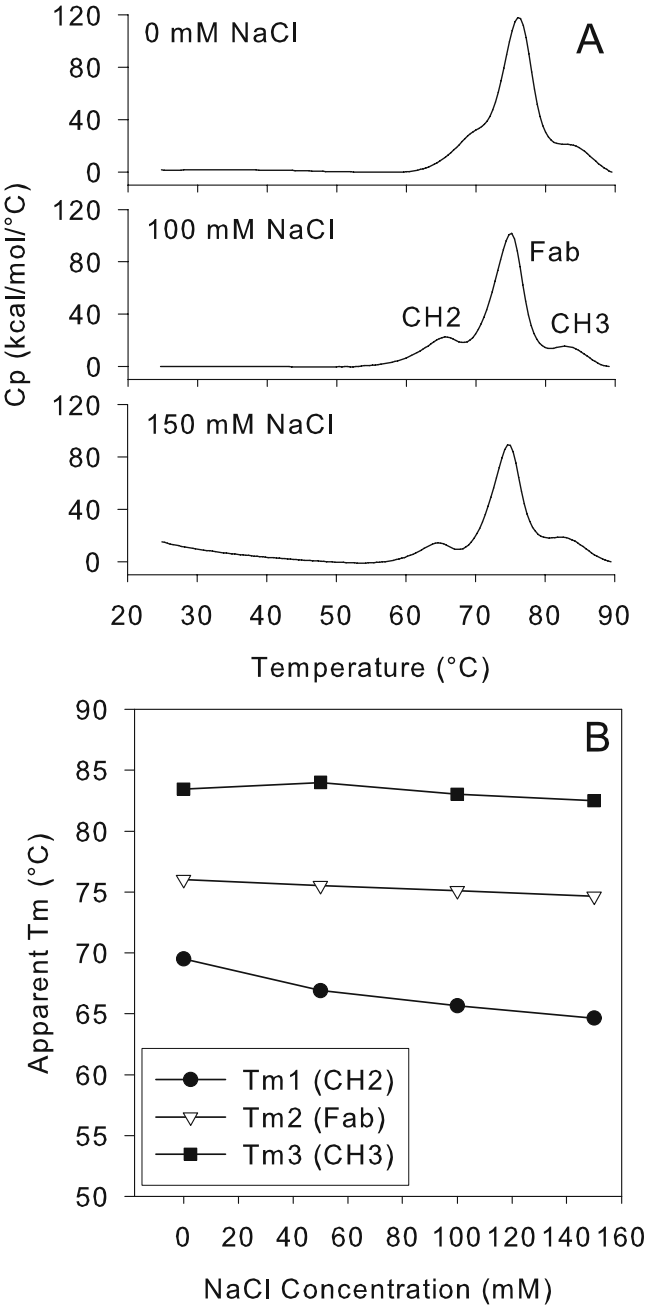


Fig. 5. DSC measurements of mAb1 melting transitions. Traces for 1.5 mg/mL mAb1 in 0, 100 and 150 mM NaCl (pH 5.0) appear in Panel A. Between 0 and 150 mM NaCl, T_{m1} displayed the largest change, and decreased with increasing salt concentration (Panel B).

gates may remain associated with the interface, however agitation-induced refreshing of the surface would expel the aggregates back into the bulk solution, increasing solution turbidity. This mechanism for aggregate formation could be enhanced through salt addition. Anion-induced charge neutralization should allow proteins to more readily partition to the surface, with the hydrophobic surface causing significantly larger aggregates than those readily formed in bulk solvent. The affinity of the protein for chaotropic anions may decrease z^* also help drive the protein to the surface. Halides with low water affinity are expected to increase in concentration at the surface (38). This model correlates well with surface tension measurements made with human serum albumin (HSA) at NaCl concentrations between 0.0 and 1.0 M (39) and with electron spectroscopy measurements made with KBr and KI (40). The surface activity of HSA was found to be maximal at its pI, but increased in the presence of NaCl. Of particular note, in solutions with pH < pI, the surface activity was significantly enhanced by NaCl addition, while at pH > pI, no significant effect is observed. This is consistent with Cl^- being competent to neutralize positive protein surface charges and Na^+ being unable to neutralize negative charges.

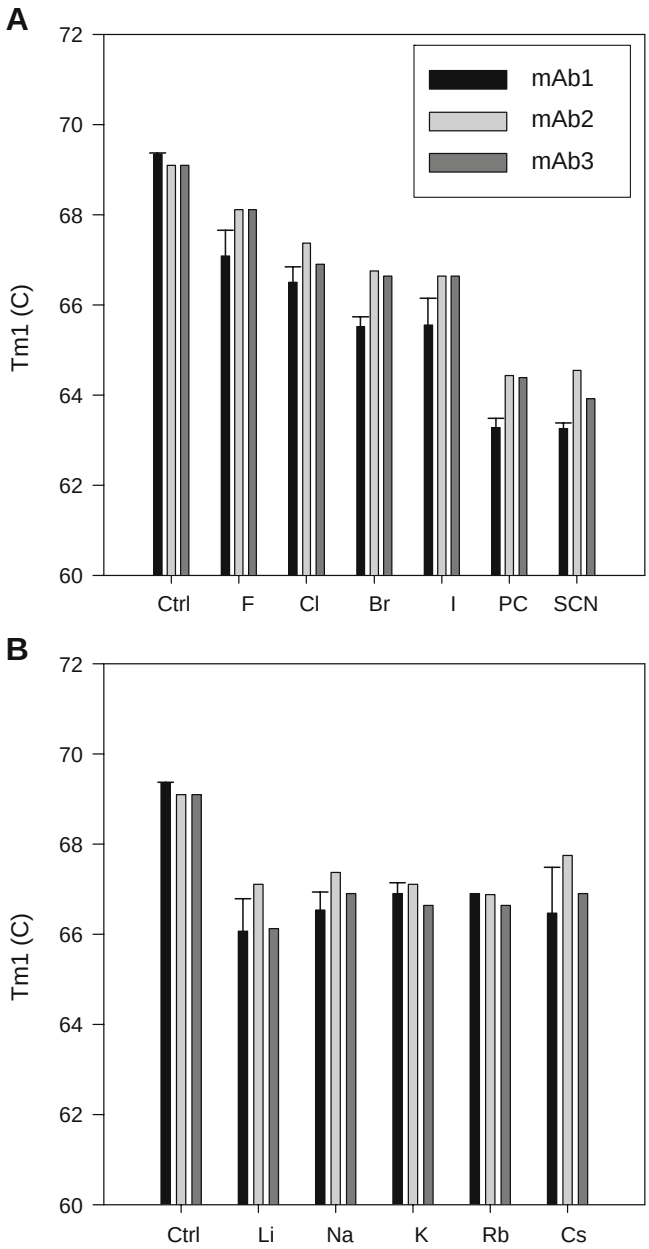


Fig. 6. The apparent T_{m1} value for mAb1, mAb2, and mAb3 was sensitive to anion (Panel A), but not cation (Panel B) identity. The tested solutions contained 10 mM acetate, and 50 mM sodium or chloride salt at pH 5.0. The control solution did not contain the 50 mM salt. The standard deviation presented for mAb1 is the result of three melts.

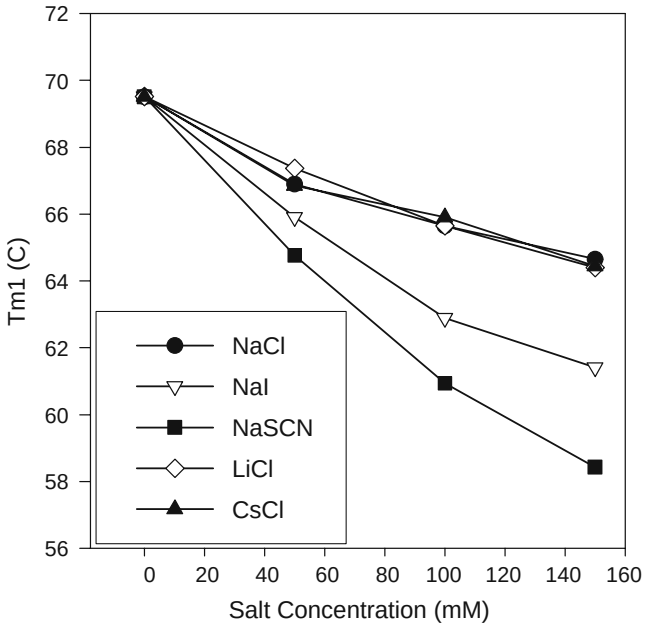


Fig. 7. The apparent T_{m1} value for mAb1 decreased with increasing salt concentration. The T_{m1} measured in the presence of salts at concentrations of 50, 100, and 150 mM in 10 mM acetate at pH 5.0. The three sodium salts (a cation series) displayed the same dependence on concentration; the chloride salts (an anion series) differed significantly in their ability to depress the measured T_{m1}.

Ions in Pharmaceutical Formulations

Salts are likely to be present in any liquid mAb formulation. They may be purposefully introduced as buffering, tonicity, or viscosity-reducing agents or may be inadvertently carried over from upstream purification processes. Our data indicate that even at milli-molar concentrations, the ions resulting from salt dissolution can have a deleterious effect on protein stability, and promote aggregate formation. The increased aggregation observed with increased salt concentration is consistent with previously published data with mono- and polyvalent buffer ions (23). In both cases, a clear correlation between increased ionic strength and increased aggregation was observed. Since the contribution to solution ionic strength increased with the valence of an ion, the use of polyvalent salts could present significant challenges for the development of aggregate-free protein therapeutics.

The use of salts for viscosity reduction can also present significant challenges. Studies conducted with high-concen-

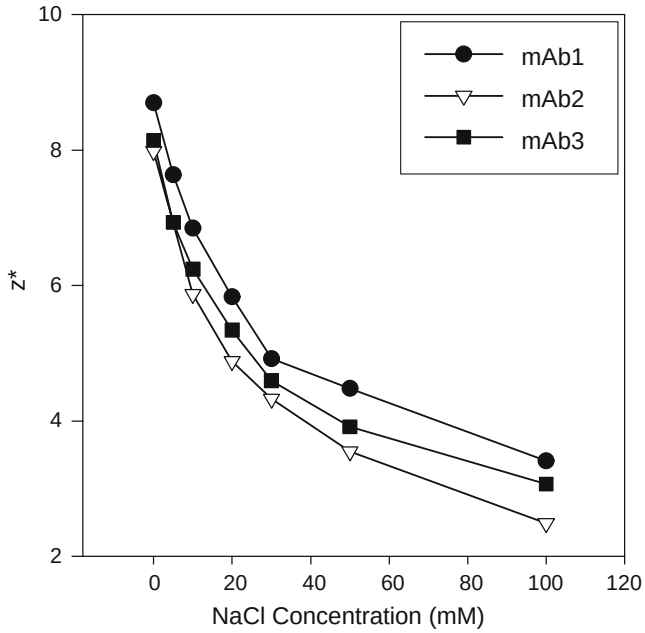


Fig. 8. Measured reduced valence as a function of acetate and NaCl concentration. The z^* of mAb1 in pH 5.0 solutions decreased with increasing NaCl concentration.

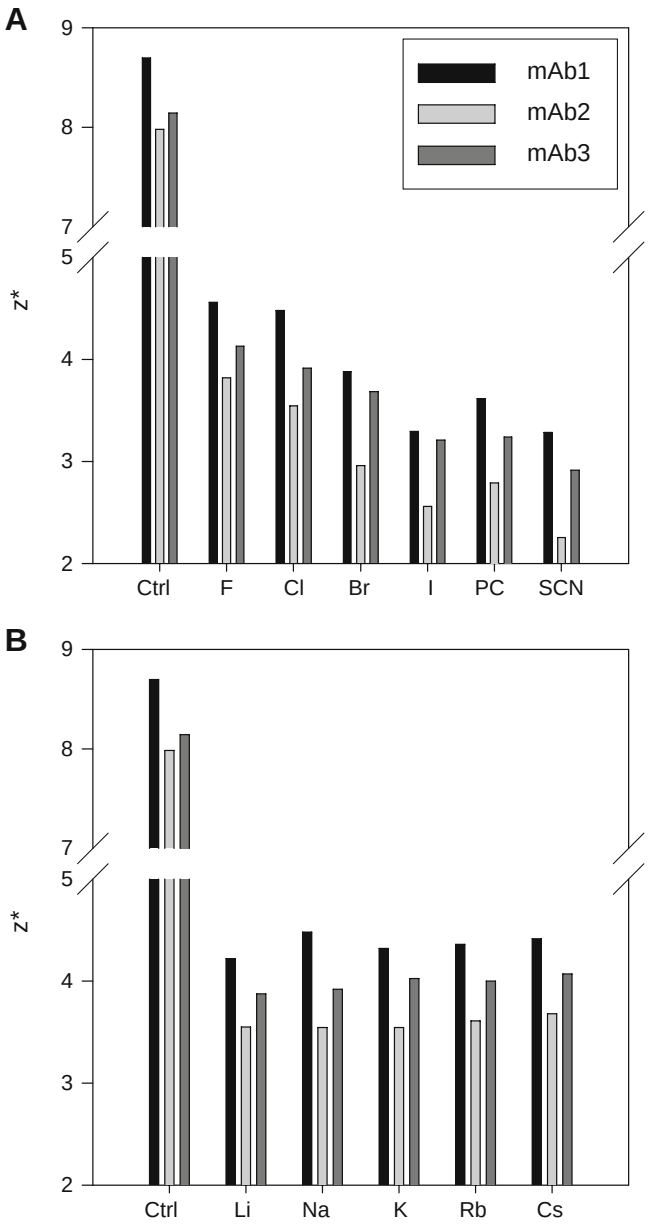


Fig. 9. Measured reduced valence as a function of anion (Panel A) or cation (Panel B) identity. The z* of mAb1, mAb2, and mAb3 in solutions containing a 50 mM salt was sensitive to anion, but not cation identity.

tration mAb solutions have shown that the solution viscosity resulting from reversible mAb self association, mediated by electrostatic interactions, was decreased with salt addition (21). Subsequent work indicated that the reduction in viscosity could be enhanced with the use of chaotropic salts, following the Hofmeister series (22). Our data, however, indicates that aggregation propensity increases with the introduction of salts to mAb solutions, also following the Hofmeister series. The differential effect of salts on reversible self association *vs*. aggregate formation suggest that these two phenomena are the result of different mechanisms, i.e. the reversible, electrostatically driven, self-association of folded protein species may not be on-pathway for the formation of aggregates. Anion binding disrupts the electrostatic associations and networks responsible for increasing solution viscosity, however it also decreases the protein surface charge. For the three antibodies in our study, this appeared to enhance the ability of the protein to engage in hydrophobic interactions, either with other proteins in the bulk solution or with the air/water interface, leading to the formation of aggregates and particles. Whether this observation can be generalized to all antibodies needs further study. Small changes in the amino acid sequence could significantly impact the aggregation interface(s), promoting or preventing aggregation even in cases where repulsive electrostatic interactions are effectively screened.

Surfactants, such as polysorbates, are frequently added to pharmaceutical protein solutions to mitigate the risk of agitation-induced aggregate formation (41), as they interfere with hydrophobic interactions at the air/water interface. While this risk can be managed to a significant degree by the addition of such compounds, their degradation products have been found to react with proteins (42,43), presenting covalent modification risks. There are also reports of polysorbate 80 causing hypersensitivity reactions (44) and anaphylaxis (45) in patients administered therapeutic protein formulations. The proteins themselves can offer some protection from aggregation at the air/water interface: the rate of agitation-induced aggregate formation, in terms of percentage conversion per unit time, has been shown to decrease with increasing protein concentration (46). This observation was rationalized as being due to the size of the air/water interface not changing as a function of protein concentration and serving as a rate limiter. Despite this apparent protection, the rate of visible aggregate formation, thought of in terms of change in aggregate mass per unit time, may still present a challenge to the development of pharmaceutical products. Development of high concentration formulations (>70 mg/mL) thus requires careful balancing of protein concentration and the addition of viscosity modifying and aggregation mitigating excipients. This emphasizes the need for an improved understanding of the effect of ions at low concentrations on protein stability.

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