Published in final edited form as:

Biotechnol Prog. 2015 ; 31(1): 268–276. doi:10.1002/btpr.2011.

A comparative study of monoclonal antibodies. 1. Phase behavior and protein-protein interactions

Rachael A. Lewus¹, Nicholas E. Levy², Abraham M. Lenhoff^{*}, and Stanley I. Sandler

Department of Chemical and Biomolecular Engineering, University of Delaware, Newark, DE 19716, USA

Abstract

Protein phase behavior is involved in numerous aspects of downstream processing, either by design as in crystallization or precipitation processes, or as an undesired effect, such as aggregation. This work explores the phase behavior of eight monoclonal antibodies (mAbs) that exhibit liquid-liquid separation, aggregation, gelation, and crystallization. The phase behavior has been studied systematically as a function of a number of factors, including solution composition and pH, in order to explore the degree of variability among different antibodies. Comparisons of the locations of phase boundaries show consistent trends as a function of solution composition; however, changing the solution pH has different effects on each of the antibodies studied. Furthermore, the types of dense phases formed varied among the antibodies. Protein-protein interactions, as reflected by values of the osmotic second virial coefficient, are used to correlate the phase behavior. The primary findings are that values of the osmotic second virial coefficient are useful for correlating phase boundary locations, though there is appreciable variability among the antibodies in the apparent strengths of the intrinsic protein-protein attraction manifested. However, the osmotic second virial coefficient does not provide a clear basis to predict the type of dense phase likely to result under a given set of solution conditions.

Keywords

monoclonal antibody; thermodynamic properties; phase equilibrium; protein interactions; aggregation

Introduction

Protein phase behavior encompasses the formation of solids, gels, or immiscible liquid phases from solutions as solvent conditions are altered. An understanding of protein phase behavior is important in a number of biological and pharmaceutical fields. A specific form of phase separation, the production of high-quality protein crystals, is the main bottleneck in determining the structures of proteins by x-ray crystallography¹. A complete understanding of phase behavior would also aid in development of a variety of pharmaceutical processing steps, especially purification, formulation and storage, and delivery^{2,3} as well as in control

^{*}Corresponding author. Phone +1 302 831 8989, lenhoff@udel.edu.

¹Current address: MedImmune, Gaithersburg, MD 20878

²Current address: GlaxoSmithKline, King of Prussia, PA 19406

of protein aggregation. Phase separation in protein solutions is also implicated in a number of pathological conditions, including the formation of cataracts ^{4,5}.

While the importance of protein phase behavior is well established, the behavior itself is still incompletely understood, particularly with regard to its sensitivity to structural differences. The focus of this work is to investigate the phase behavior of monoclonal antibodies (mAbs) and to develop a framework to understand those results based on the colloidal phase diagram for macromolecules that interact over length scales that are short relative to the macromolecular size, as has been done previously for a set of model proteins ⁶ and for a single mAb ⁷. Solution conditions that can be changed to alter phase behavior include the salt type and concentration, pH, temperature, and the type and concentration of any additives ^{8–10}. Determining the phase behavior is complicated by the large parameter space, the complex interactions among the protein and the additives at different solution conditions, and difficulties in characterizing the dense phases that are present. In addition, the time to attain phase equilibrium can be long.

The origins of trends in protein phase behavior are expected to be correlated with the magnitude and nature of protein-protein interactions, which are most frequently characterized in terms of the second osmotic virial coefficient (B_{22}). B_{22} is an averaged quantity reflecting underlying net interactions that are highly anisotropic; it is related to the underlying potential of mean force W of pairwise protein-protein interactions by ¹¹

$$B_{22} = \frac{1}{16MW^2\pi^2} \int_{\Omega} \left[\frac{1}{3}r_c^3 - \int_{r_c}^{\infty} (e^{-W/kT} - 1)r_{12}^2 dr_{12} \right] d\Omega \quad (1)$$

where MW is the protein molecular weight, kT is the thermal energy, r_{12} is the center-to-center distance, with r_c the value at contact, and Ω represents the five-dimensional angular coordinates defining the relative orientations of the two molecules. Note that a net attractive potential of mean force results in a negative value of B_{22} .

While the average interaction has been observed to be related to liquid-liquid phase separation ¹², the anisotropy is important in crystallization ¹³. Models of protein solubility and the liquid-liquid phase boundary that account for anisotropy in a simple way are able to quantitatively describe experimental data when isotropic models fail ^{14,15}. Detailed models of bimolecular protein interactions indicate that net attraction measured by B_{22} stems disproportionately from a few highly attractive configurations exhibiting surface complementarity ^{11,16}. This observation is useful in explaining why B_{22} can be effective for correlating with the propensity of a protein to crystallize. Since different distributions of interactions can lead to similar B_{22} values, correlations are not always completely informative. Nevertheless, B_{22} can be useful for characterizing protein interactions, and it is used here to aid in understanding the observed phase behavior.

The goals of this work are to characterize the phase behavior and interactions of a number of antibodies over a range of solution conditions, including exploring the effects of multiple

salts, solution pH, and the additive polyethylene glycol (PEG). The phase behavior and interactions are compared among the antibodies to gain an improved understanding of the extent of the similarities and differences among antibodies. The supporting measurements of B₂₂ were performed to gain insight into the utility of such values for predicting antibody phase boundaries.

The motivation for a comparative study of the phase behavior and interactions of different mAbs lies ultimately in the ubiquitous and growing development of this class of therapeutics. In the course of process development as well as for purposes of structural characterization, appreciable differences have been observed among different mAbs in such properties as viscosity^{17–20} and propensity to crystallize²¹. Such measurements can generally be expected to be related to the nature of mAb self-interactions in solution, which in turn are manifested in phase behavior measurements more generally.

Previous related work dealt with the behavior of a single monoclonal antibody, IDEC-152, and its response to varying solution pH and the addition of sulfate salts and the polymer PEG⁷; other conditions have also been explored for this mAb²². The solvent conditions used were chosen because they give rise to solvent and depletion effects expected to promote attractive interactions generally, as distinct from highly specific local electrostatic attraction that is thought to give rise to salting-in at low salt²³, corresponding to conditions under which mAb interactions have been studied extensively^{17,18,20}. Here our previous study⁷ is extended to encompass the behavior of seven additional monoclonal antibodies in order to develop a more general understanding of antibody behavior for applications throughout protein processing.

The mAbs studied were generously provided by four different companies, and while all are IgGs, limited additional characteristics were provided other than the class. However, in view of the origins in different companies and known differences in class, the group of molecules studied is expected to cover a representative range of structural and functional variability, and hence to provide a reasonable measure of the variability expected to be encountered in mAbs under development. One caveat, though, is that a subset of the mAbs studied were selected by the originating companies because of one or other anomaly observed during processing or elsewhere.

Materials and Methods

Materials and Solutions

Ammonium sulfate (A2939), lithium sulfate (L6375), bis-tris (B7535), sodium formate (S2140), polyethylene glycol (PEG) (average molecular weight 3350, P4338), glutaraldehyde (G5882), ethanolamine (E9508), and EDTA (E9884) were purchased from Sigma (St. Louis, MO). Sodium chloride (S271) and sodium acetate (S209) were purchased from Fisher (Pittsburgh, PA). Solutions were made using DI water further purified using an EMD Millipore Milli-Q® system (Billerica, MA) and pH-adjusted using small amounts of concentrated sodium hydroxide or hydrochloric acid solutions. The pH was maintained at 7.0 by 5 mM bis-tris, at 5.0 by 10 mM sodium acetate, or at 3.0 by 10 mM sodium formate.

IDEC-151, an IgG4, and IDEC-152, an IgG1, were donated by Biogen Idec (San Diego, CA); both are human antibodies with variable regions derived from primates. IDEC-151 was delivered lyophilized and was rehydrated to a concentration of 69 mg/mL, and IDEC-152 was delivered in a formulation buffer at 49 mg/mL. Mabs A, B and C are IgG1s that were donated by Genentech (South San Francisco, CA). They have theoretically calculated pIs of 9.25, 7.85 and 9.35 and were delivered in formulation buffers at 3.2, 22 and 100 mg/mL, respectively. Mabs D and D_M, provided by Amgen Inc. (Seattle, WA), are closely related IgG2s that differ only in that two Arg residues near the complementarity-determining regions (CDRs) of mAb D are mutated to an Ala and a Thr in mAb D_M. They were provided in formulation buffer at 150 and 32.2 mg/mL, respectively. Finally, Milli004 (EMD Millipore, Bedford, MA) is an IgG that was provided in buffer at 60 mg/mL.

The purity of each mAb was checked by gel electrophoresis and ion exchange chromatography and all were used without further purification. Buffer exchanges were carried out in Slide-A-Lyzer cassettes (66810, 66380) from Pierce Biotechnology (Rockford, IL) by dialyzing three times against the final buffer for at least 4 hours each at $4 \pm 1^\circ\text{C}$. Solutions were concentrated using Amicon® ultracentrifugal filter units (UFC8-030-96) from EMD Millipore. Protein concentrations were determined from UV absorbance at 280 nm, measured using a Perkin-Elmer Lambda 4B spectrophotometer. The extinction coefficients used were provided by the manufacturers or calculated from dilution curves of solutions of known concentration.

Experimental Procedures

The phase behavior of mAb solutions was determined by microbatch experiments as described in detail previously⁷. Such measurements allow observation of the short- and long-time phase behavior for differing protein concentrations and solvent conditions (pH, salt type and concentration, PEG concentration) in individual wells on microtiter plates. Changes in phase behavior over the range of conditions covered allow the locations of phase boundaries to be determined.

Self-interaction chromatography (SIC) was used to determine the second osmotic virial coefficient (B_{22}). This method has previously been shown to provide results that agree in general with those obtained using other methods of measuring B_{22} ^{24–26}. The procedures used for SIC measurements on monoclonal antibodies are described in detail elsewhere⁷. Phase behavior and SIC experiments were performed at room temperature.

Results and Discussion

The eight mAbs investigated have been studied in somewhat different levels of detail. IDEC-152 was characterized to the greatest extent⁷ and provides a frame of reference for the other seven. Of the latter, Mab A and Mab B were characterized to a degree similar to that for IDEC-152, and they are first discussed separately below in view of the appreciable differences in their behavior. Descriptions of the dense phases obtained are provided separately in the Supplementary Materials. The remaining five mAbs were studied somewhat less extensively, particularly with regard to the types of dense phases obtained, so

results for them are presented in the form of comparisons that are made among phase diagrams and B₂₂ measurements for all eight antibodies studied.

Phase Behavior and Interactions of Mab A

Phase Behavior in Salt Solutions—Mab A exhibits extremely strong protein-protein attraction under a variety of solution conditions. As a result, phase separation is observed over a wide range of sulfate salt concentrations as well as in solutions of sodium chloride. The phase diagrams for Mab A in ammonium and lithium sulfate solutions at pH 7.0 and 5.0 are shown in Figure 1. Two sets of data are shown in each case. The first, at higher salt or protein concentrations, represents the boundary beyond which phase separation is observed instantaneously upon mixing; this line is assumed to represent the spinodal ^{6,7}. The second set of data is based on observations after two months and is assumed to reflect equilibrium conditions, although there may be some uncertainty regarding these data because of the possibility of effects such as evaporation and structural changes after an extended period at room temperature. Recovery and characterization of mAbs from dense phases such as those studied here is described elsewhere ²⁷.

The behavior shown in Figure 1 is qualitatively similar to that observed for IDEC-152 at similar solution conditions⁷. Steep boundaries denoting instantaneous phase separation indicate that beyond a certain salt concentration, the protein does not remain soluble. In sulfate salt solutions, much of the phase space exhibits formation of dense phases. Also, a reduction of the pH from 7.0 to 5.0 causes a decrease in attraction, presumably due to increased electrostatic repulsion, as Mab A has a pI of 9.25. Lastly, the differences between the results for the ammonium and lithium salts indicate that the cation effect is significant, as was observed in IDEC-152 solutions.

Beyond the similarities, two major differences relative to IDEC-152 are observed in the phase diagram: the locations of the boundaries are at much lower salt concentrations for Mab A, indicating strong protein-protein attraction, and the kinetics, which are not shown, are rapid, with most dense phases forming in under one week. This is significantly different from IDEC-152 phase separation, which took over a month in many cases⁷.

In lithium and ammonium sulfate solutions, the most common dense phases were gels, especially gel networks. Poorly formed crystalline phases also formed under some conditions in sulfate salts. The dense phases are described in greater detail in the Supplementary Material.

Mab A was the only protein investigated in this work that showed phase separation in sodium chloride solutions. Sodium chloride is a significantly weaker salting-out agent than the sulfate salts, based on the Hofmeister series ²⁸; however, it is effective at salting out Mab A, which, as noted above, displays more attractive protein-protein interactions than most of the other antibodies studied here. The phase diagram for Mab A in sodium chloride solutions is shown in Figure 2. The instantaneous phase boundary occurs at significantly higher salt concentrations than for solutions of either sulfate salt, as expected.

The equilibrium curve shows that all of the solution conditions examined with sodium chloride at pH 7.0 resulted in phase separation; the dense phases formed were primarily crystalline. Further, the solutions phase-separated quickly, in less than 9 days in most cases. The exception is the lowest salt and protein concentration combination, 0.25 M NaCl with 5 mg/mL Mab A, which took more than one month to crystallize. Since buffered solutions of Mab A without added salt are stable at pH 7.0 for all protein concentrations studied, reducing the salt concentration must eventually lead to a one-phase region of the phase diagram, but one was not observed for solution conditions investigated here, indicating that that portion of the equilibrium phase boundary is steep.

Effect of PEG—The effect of PEG 3350 on phase behavior was examined for solutions of Mab A in all three salts at pH 7.0. In Figure 3 the results of the instantaneous phase separation for 2, 4, and 6 wt % PEG 3350 are compared to the behavior without PEG. For lithium sulfate and sodium chloride solutions, the results follow the trend expected for depletion effects, exhibiting phase separation at progressively lower salt concentrations as the polymer concentration is increased. In ammonium sulfate solutions, little change is observed until 6 wt % PEG, where there is a clear effect consistent with depletion interactions. This apparent threshold for the onset of a measurable PEG effect may be similar to the repulsive effect, inconsistent with depletion effects, observed for IDEC-152 in solutions with low concentrations of PEG⁷, though no clear shift to higher salt concentrations like that found in that case is observed here.

Protein-Protein Interactions—Although the phase behavior at all conditions studied indicates strong protein-protein attraction compared to IDEC-152, quantitatively characterizing this is difficult. Self-interaction chromatography was successful only for a small number of runs before the column fouled and clear peaks were no longer observed. We believe that the interactions among both immobilized and unbound protein molecules were attractive enough to precipitate a dense phase in the column, which recruited protein from new injections or physically blocked the column, and made any subsequent measurements invalid. Attempts to clean the column were not successful, and similar problems were encountered with a second column, leading to a conclusion that self-interaction chromatography measurements would be unsuccessful for Mab A. SIC and static light scattering methods were not successful for measuring Mab A B_{22} values because Mab A forms high concentrations of oligomers in the solution conditions of interest²⁹.

Although these difficulties precluded measurement of full sets of B_{22} values, several measurements were completed with ammonium sulfate, lithium sulfate, and sodium chloride solutions at pH 7.0 before the fouling occurred. More negative B_{22} values, the result of stronger attraction, were observed for Mab A than for IDEC-152 in solutions of all salts. Since the internal structure of the column and the resulting phenomena may have been affected before the irregularities were externally observable, these results should be considered primarily qualitative

Static light scattering could not be used to measure interactions due to the oligomerization behavior of Mab A, which is discussed elsewhere¹⁰. The change in scattering due to the presence of oligomers can not readily be deconvoluted from the effect of interactions.

Phase Behavior and Interactions of Mab B

Behavior in Salt Solutions—The B_{22} values of Mab B in sulfate salt solutions at pH 7.0 and 5.0 (Figure 4) show increasingly attractive interactions with increasing salt concentration, with the most negative (attractive) B_{22} values similar to those seen for IDEC-152⁷. The trends are those that would be expected based on the Hofmeister series and a basic pI, although the pI of Mab B is not known. Specifically, lithium sulfate causes stronger attraction than ammonium sulfate and for both salts the shift to lower pH causes a decrease in attraction. This is consistent with classical electrostatic interactions despite the fact that such interactions should be essentially completely screened at the salt concentrations investigated. Similar results were previously observed for model protein systems and were attributed to the hydration of acidic groups⁹, although other effects such as ion binding may also play a role. The effect of pH in ammonium sulfate solutions is small, as is the cation effect at pH 5.0, resulting in similar B_{22} values at all conditions except in Li_2SO_4 at pH 7.0, where more negative values are seen.

The instantaneous phase boundaries of Mab B, shown in Figure 5, exhibit the dependence on salt and pH that would be expected from the B_{22} curves. However, all of the curves lie close together, indicating that the increased attraction in lithium sulfate solutions at pH 7.0 has a minimal effect on the location of the instantaneous phase boundary. The equilibrium boundaries (Figure 5), measured after two months, do not follow the same order as expected from the instantaneous boundaries and B_{22} curves, an unexpected result. The pH 5.0 curves appear at the lowest salt concentrations, followed by $(\text{NH}_4)_2\text{SO}_4$ at pH 7.0 and Li_2SO_4 at pH 7.0.

A number of explanations for this behavior are possible. One is that the solutions at pH 7.0 had not reached equilibrium within the two months allotted, but that the solutions at pH 5.0 had. This is supported by the rapid phase separation observed at pH 5.0 and the fact that new dense phases were observed between the 6 and 8 weeks time points at pH 7.0 for solutions of both salts, indicating that equilibrium may not have been reached. Unfortunately, longer time points were not experimentally tractable because evaporation was becoming significant despite the layer of silicone oil.

The difference in dense phases formed may provide another explanation for these results. Solutions of lithium sulfate at pH 7.0 resulted in a gel phase, while all the others resulted in a solid, which may be crystalline. If it is crystalline, the equilibrium boundary would be a crystal nucleation line, not the dilute branch of the liquid-liquid phase boundary^{6,7} in the framework of the theoretical colloidal phase diagram. The crystal nucleation boundary lies above the fluid line in equilibrium with the crystal, i.e., the solubility, so it is reasonable that the liquid-liquid boundary for lithium sulfate solutions at pH 7.0 would occur at higher salt concentrations than the crystal nucleation boundaries for the other three conditions. Different boundaries on the colloidal phase diagram and incomplete equilibrium may therefore both play a role.

Effect of PEG—The effect on the phase behavior of Mab B of adding PEG 3350 was examined in solutions of both sulfate salts at pH 7.0. The instantaneous boundaries for 2 to 6 wt % PEG are compared to those for the case without polymer in Figure 6. The addition of 2

wt % PEG causes a shift to slightly higher salt concentrations, indicating an increase in repulsive interactions. Increasing the PEG concentration to 6 wt % results in an increase in attraction consistent with that expected from depletion effects. The intermediate polymer concentration has different effects dependent on the salt type. This behavior is similar to that found for IDEC-152⁷ and for Mab A (Figure 3) in ammonium sulfate solutions. The equilibrium boundaries (not shown) exhibit a slight shift to lower salt concentrations for all polymer concentrations, indicating that the repulsive effect at low polymer concentrations may affect only the instantaneous boundary.

Generally, Mab B behaves similarly to IDEC-152⁷ in terms of the shape and location of the phase boundaries, although these are shifted to slightly higher salt concentrations for Mab B. The effect of PEG 3350 is also similar; low polymer concentrations cause increased repulsion and the trend expected from depletion effects is recovered as the polymer concentration is increased. However, the dense phases observed are quite different for the two antibodies (see Supplementary Materials).

Comparison of Eight Monoclonal Antibodies

Phase Boundaries and Interactions—The results for the two mAbs discussed above and those for IDEC-152⁷ have been augmented by limited measurements on five additional mAbs. These measurements include B_{22} values and instantaneous phase boundaries for different salt types and concentrations.

The response to changes in salt type in all cases is consistent with the Hofmeister series and previous studies with model proteins and IDEC-152^{7,30,31}. Sodium chloride results in the least attraction for the mAbs studied (Figure 7), but Mab A and Milli04 display attractive interactions even at low salt concentrations, while at very high salt concentrations (> 3 M) the interactions of IDEC-151, IDEC-152 and Mab B are attractive. Mab A was the only antibody to phase-separate in NaCl within the range of solution conditions tested here, despite the similar interactions measured for Milli04. Phase separation has been observed for IDEC-152 in more concentrated NaCl solutions²⁶. Previous work has shown that some mAbs in chloride salt solutions tend to become more flexible and allow different residues to become accessible³², which may affect the intermolecular interactions and phase behavior.

As expected sulfate salts are much stronger salting-out agents, and all eight antibodies phase-separate in concentrated solutions of either ammonium or lithium sulfate. Figure 8 shows instantaneous phase boundaries in addition to the B_{22} values, and both indicate that the interactions are much more strongly attractive than in NaCl. The cation effect is also in line with predictions, with lithium sulfate causing stronger attraction than ammonium sulfate in most cases. The equilibrium phase boundaries exhibit the same trends as the instantaneous boundaries but are more difficult to distinguish from each other because they are shallow, so they are not shown. Although the relative effects of the salts are similar, the actual strengths of the interactions depend on the individual proteins. The protein-protein interactions for Mab A and Milli004 are significantly more attractive than those for any of the other proteins, and Mab D has the weakest attractions in all solution conditions explored. IDEC-151, IDEC-152, Mab B, Mab C and Mab D_M have similar interactions and phase boundaries, which are shifted to higher salt concentrations than those for Mab A and

Milli004. In ammonium sulfate solutions the B_{22} data for these five mAbs are almost identical, while the corresponding phase boundaries are spread over a range of about 0.25 M ammonium sulfate. In lithium sulfate solutions the B_{22} values and phase boundaries are in the same relative positions, but they cover a larger range of salt concentrations. There is a general agreement between interaction strength and phase boundaries, but it is not possible to predict the boundary location knowing only the interaction strength based on the value of B_{22} . However, the relationship between phase behavior and interactions is consistent for each protein in different salt types. Once the interactions and phase behavior of a given mAb have been measured for a single salt, the phase behavior for that mAb in a different salt can be reasonably estimated from only the measured B_{22} values, or vice versa.

The effect of pH on IDEC-152 solutions showed an unexpected trend in that interactions at pH 3.0 were more attractive than those at pH 5.0⁷; the trend was observed in both the phase boundaries and B_{22} values. Possible reasons for this were discussed previously⁷, including partial unfolding at low pH and steric effects caused by hydration of acidic amino acids. A similar relationship between pH and interactions is seen for Mabs D and D_M , as reflected in the instantaneous phase boundaries at constant salt concentration shown in Figure 9. However, Mabs A and B follow the expected trend for a molecule with a basic pI, with the interactions becoming more repulsive as the net charge increases at lower pH, further from the isoelectric point. This indicates that the effects causing the reversal of this trend for IDEC-152, Mab D and Mab D_M do not play a comparable role here, potentially due to differences in distributions of charged amino acids on the regions of the protein surface that most strongly affect interactions. Although the global effect of changing pH on net charge is understood, the distribution of those amino acids in the Mab and the effect of the local environment on their pK_a values may vary significantly from protein to protein. In addition, as noted earlier, classical electrostatic effects are not expected to be dominant at such high salt concentrations, so other effects such as hydration and ion binding may also be implicated. For all of these effects the anisotropy plays a significant role and makes *a priori* prediction of pH effects difficult.

B_{22} provides a highly averaged measure of protein-protein interactions, but one that can be strongly influenced by interactions in only a small subset of pairwise molecular configurations¹¹. The large difference in the interaction patterns for Mab D and Mab D_M confirms the importance of the contribution of changes in a small number of residues, in this case two residues near the CDR. Similarly, the much stronger protein-protein attraction seen for Mab A and Milli004 may be due to relatively small structural differences compared to molecules with less extreme interaction patterns. Although detailed structural information is unavailable for the eight mAbs studied, differences in the IgG class and among the molecular templates used by different companies may give rise to more extensive structural differences. However, Mab A is of the same IgG class and provided by the same company as Mabs B and C, which show much weaker attraction.

The B_{22} curves correlate qualitatively with the locations of the phase boundaries, although the correlation is weaker when comparing different antibodies than when comparing different solution conditions for a single antibody. Although inconsistencies in the order predicted by the interactions have not been observed, there are cases where very similar B_{22}

values do not correlate well with the corresponding instantaneous phase boundaries. That the correlation between B_{22} values and phase boundaries is not closer is presumably due to the role of specific local interactions in driving phase separation, resulting in a controlling role for an ensemble of pairwise configurations that is much more limited than the global averaging over all configurations represented in B_{22} . This result indicates that a much better understanding of local interactions is necessary for accurate prediction of phase behavior, although B_{22} values certainly appear effective in capturing overall trends.

Dense Phases—Dense phases were characterized for IDEC-151, Mab A and Mab B in addition to the results reported previously for IDEC-152⁷. In contrast to the clear trends in the interactions and phase boundaries described above, the dense phases formed vary widely and evolved with time, as discussed in detail in the Supplementary Material. For IDEC-151 and 152, gel drops are most common. Mab A and B both form gel networks over a wide range of solution conditions. Mab A crystallizes easily, especially in NaCl solutions. Mab B forms “fuzz balls”, which appear to be crystalline due to their birefringence, but are poorly formed. These structures may be similar to previously reported lysozyme “sea urchins”³³, although as the informal names suggest, the structures seen here appear to have more substantial cores and less extensive protruding crystalline needles. The type of dense phase observed is not correlated to B_{22} values, which reflect two-body interaction strengths. Multi-body interactions might be an important factor in the development of various dense phase types because most protein molecules in a dense phase are involved in short-range interactions with multiple neighbors. B_{22} values are insufficient to describe protein-protein interactions in dense phases because of the high local protein concentrations³⁴.

The dense phase behavior as a result of the addition of PEG is similar to that of increased salt concentration in most cases. The exception is IDEC-152, where crystals are observed only in solutions containing PEG 3350. Also, high concentrations of polymer and salt frequently result in formation of concentrated liquid drops, generally in the presence of a second dense phase that is similar to the dense phase observed without polymer. The liquid drop may be related to the polymer-salt phase separation that can occur without protein present. It is difficult to determine the distribution of salt and protein between the drops and the bulk solution; however, the presence of the second dense phase suggests that the protein remains primarily outside the drops. Overall, few similarities are observed in the dense phases among the antibodies. However, IDEC-152, Mab A, and Mab B all crystallize, if fuzz balls are considered crystalline.

The conditions that yield crystals are examined in the context of George and Wilson's crystallization slot, according to which B_{22} values between -1 and -8×10^{-4} mol mL/g² are correlated with a propensity to crystallize³⁵. The results in this work (Figure 10) indicate that in most cases at least part of the salt concentration range yields measured B_{22} values within the crystallization slot for solution conditions that yield putative crystalline phases, but that in many cases crystallization also occurs for more negative B_{22} values. Therefore, although many conditions that yield crystals are within the crystallization slot, the correlation is not strong for these antibodies.

Large proteins generally do not exhibit the very negative B_{22} values found for the antibodies studied here^{36–38}, since expressing B_{22} in units of ml mol/g^2 involves division by the square of the molecular weight. For this reason, crystallization conditions for large molecules often occur at the less attractive end of the crystallization slot. That the values of B_{22} found here include a significant number that are even more negative than the range spanned by the crystallization slot, and that some of the solutions involved are stable nonetheless, is therefore a surprising result. An alternative representation of the crystallization slot, to avoid artifacts of protein size, gives a range of approximately 3 to -10 in terms of a dimensionless form of B_{22} , with B_{22} scaled relative to the excluded-volume contribution³⁶. However, the definition of the excluded volume used there is essentially a variant of the molecular volume and therefore smaller than the usual excluded volume³⁹ by a factor of about 6.7. Allowing for this gives rise to the range shown by the dashed lines in Figure 10, which is very narrow and lies entirely above the George and Wilson crystallization slot and is therefore in even stronger disagreement with the nominal crystallization slot range. The very negative values of B_{22} found here may be related to the high salt concentration in these solutions, which was not found in the crystallization conditions for the large proteins used to define the dimensionless crystallization slot. It is also possible that the values of B_{22} found here are exaggerated by an experimental artifact, though the trends are validated by their correlation with phase boundaries.

A possible structural contribution to the anomalously negative B_{22} values is the distinctive shape of IgG molecules. Since the interactions contributing to B_{22} are very short-ranged in nature, the integral within the brackets in eqn. (1) is dominated by the contributions at separation distances r_{12} close to the contact separation r_c , so that the multivariable integration over r_{12} and Ω is effectively a surface integral. Because of their peculiar shape, IgG molecules have surface areas larger than those of globular molecules of similar molecular weights; calculations using the Molecular Surface Package⁴⁰ indicate that IgG surface areas are more than 50% higher than expected for proteins of this molecular weight⁴¹. This can appreciably affect the value of B_{22} , though not necessarily to the extent seen in the measured values relative to those expected for such large molecules.

Conclusions

The antibodies studied here are strongly salted-out by sulfate salts, and follow the Hofmeister series in their response to changes in salt type. Generally the behavior of IDEC 152, IDEC 151, Mab B, Mab C and Mab D_M appeared similar, while Mab A and Milli04 behaved differently, exhibiting stronger attraction and a greater tendency to crystallize. Mab D showed much weaker attractions. The hypervariable region may be responsible for these differences, or the subclass of Mab A and Milli04 may play a role. Charged residues near the CDR play a major role in interactions, as evidenced by the behavior of Mab D and Mab D_M.

Correlations between interaction measurements, in terms of B_{22} , and phase boundaries for salt type and protein type suggest that the general behavior and trends of additional antibodies can be predicted based on a small amount of information. Unfortunately, those correlations are limited to phase boundaries and do not extend to the prediction of dense phase type.

The anisotropy of antibodies appears to play a significant role in their phase behavior and our ability to develop predictions and correlations of this behavior from B_{22} data or other easily measurable quantities. The anisotropy of the charge distribution makes comparing the pH effect on antibodies difficult, although the B_{22} values correlate well with this behavior. While fluctuations leading to nucleation events can be affected by averaged interactions, phase separation, and especially crystallization, is believed to be controlled by specific attractions, while B_{22} values represent averaged interactions. Currently no measurement techniques exist that can provide direct information on the anisotropic contributions to protein-protein interactions, and any conceptual ideas that might account for these contributions would require very detailed structural information to assess reliably. However, the anisotropic effects are the most likely explanation for poor interaction-phase boundary correlations, and developing an approach to account for this aspect of the behavior is vital to predictive methods for antibody phase behavior. These anisotropic effects are further explored in two companion papers that examine oligomerization trends and the contributions of mAb subunits to interaction behavior.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful for support from the National Science Foundation under grant number BES-0519191. We also thank Amgen Inc., Biogen Idec, Genentech and EMD Millipore for generously providing the mAbs studied, without which this work would not have been possible.

Literature Cited

1. Tessier PM, Vandrey SD, Berger BW, Pazhianur R, Sandler SI, Lenhoff AM. Self-interaction chromatography: a novel screening method for rational protein crystallization. *Acta Crystallogr Sect D Biol Crystallogr*. 2002; 58:1531–1535. [PubMed: 12351855]
2. Yang MX, Shenoy B, Disttler M, Patel R, McGrath M, Pechenov S, Margolin AL. Crystalline monoclonal antibodies for subcutaneous delivery. *Proc Natl Acad Sci U S A*. 2003; 100:6934–6939. [PubMed: 12782786]
3. Basu SK. Protein crystals for the delivery of biopharmaceuticals. *Expert Opin Biol Ther*. 2004; 4:301–317. [PubMed: 15006725]
4. Chen YC, Reid GE, Simpson RJ, Truscott RJ. Molecular evidence for the involvement of alpha crystallin in the colouration/crosslinking of crystallins in age-related nuclear cataract. *Exp Eye Res*. 1997; 65:835–840. [PubMed: 9441707]
5. Lashuel HA, Hartley D, Petre BM, Walz T, Jr PTL, Turner J, King JC, Lachlan-Cope TA, Jones PD. Amyloid pores from pathogenic mutations. *Nature*. 2002; 418:4–5.
6. Dumetz AC, Chockla AM, Kaler EW, Lenhoff AM. Protein phase behavior in aqueous solutions: crystallization, liquid-liquid phase separation, gels, and aggregates. *Biophys J*. 2008; 94:570–583. [PubMed: 18160663]
7. Lewus RA, Darcy PA, Lenhoff AM, Sandler SI. Interactions and phase behavior of a monoclonal antibody. *Biotechnol Prog*. 2011; 27:280–289. [PubMed: 21312375]
8. Tessier PM, Johnson HR, Pazhianur R, Berger BW, Prentice JL, Bahnson BJ, Sandler SI, Lenhoff AM. Predictive crystallization of ribonuclease A via rapid screening of osmotic second virial coefficients. *Proteins*. 2003; 50:303–311. [PubMed: 12486724]

9. Dumetz AC, Chockla AM, Kaler EW, Lenhoff AM. Effects of pH on protein-protein interactions and implications for protein phase behavior. *Biochim Biophys Acta*. 2008; 1784:600–610. [PubMed: 18258214]
10. Lewus RA, Levy NE, Sandler SI, Lenhoff AM. A comparative study of monoclonal antibodies. 2 Oligomerization. preparation.
11. Neal BL, Asthagiri D, Lenhoff AM. Molecular origins of osmotic second virial coefficients of proteins. *Biophys J*. 1998; 75:2469–77. [PubMed: 9788942]
12. Rosenbaum D, Kulkarni A, Ramakrishnan S, Zukoski CF. Protein interactions and phase behavior: Sensitivity to the form of the pair potential. *J Chem Phys*. 1999; 111:9882–9890.
13. Curtis RA, Lue L. A molecular approach to bioseparations: Protein – protein and protein – salt interactions. *Chem Eng Sci*. 2006; 61:907–923.
14. Haas C, Drenth J, Wilson WW. Relation between the solubility of proteins in aqueous solutions and the second virial coefficient of the solution. *J Phys Chem B*. 1999; 103:2808–2811.
15. Lomakin A, Asherie N, Benedek GB. Aeolotropic interactions of globular proteins. *Proc Natl Acad Sci U S A*. 1999; 96:9465–9468. [PubMed: 10449715]
16. Neal BL, Asthagiri D, Velev OD, Lenhoff AM. Why is the osmotic second virial coefficient related to protein crystallization? *J Cryst Growth*. 1999; 196:377–387.
17. Yadav S, Laue TM, Kalonia DS, Singh SN, Shire SJ. The influence of charge distribution on self-association and viscosity behavior of monoclonal antibody solutions. *Mol Pharm*. 2012; 9:791–802. [PubMed: 22352470]
18. Connolly BD, Petry C, Yadav S, Demeule B, Ciaccio N, Moore JMR, Shire SJ, Gokarn YR. Weak interactions govern the viscosity of concentrated antibody solutions: high-throughput analysis using the diffusion interaction parameter. *Biophys J*. 2012; 103:69–78. [PubMed: 22828333]
19. Saito S, Hasegawa J, Kobayashi N, Kishi N, Uchiyama S, Fukui K. Behavior of monoclonal antibodies : Relation between the second virial coefficient (B_2) at low concentrations and aggregation propensity and viscosity at high concentrations. *Pharm Res*. 2012; 29:397–410. [PubMed: 21853361]
20. Yadav S, Sreedhara A, Kanai S, Liu J. Establishing a link between amino acid sequences and self-associating and viscoelastic behavior of two closely related monoclonal antibodies. *Pharm Res*. 2011; 28:1750–1764. [PubMed: 21626060]
21. Trilisky E, Gillespie R, Osslund TD, Vunnum S. Crystallization and liquid-liquid phase separation of monoclonal antibodies and Fc-fusion proteins: Screening results. *Biotechnol Prog*. 2011; 27:1054–1067. [PubMed: 21656920]
22. Ahamed T, Esteban BNA, Ottens M, van Dedem GWK, van der Wielen LAM, Bisschops MAT, Lee A, Pham C, Thömmes J. Phase behavior of an intact monoclonal antibody. *Biophys J*. 2007; 93:610–9. [PubMed: 17449660]
23. Tanford, C. *Physical Chemistry of Macromolecules*. New York: Wiley; 1961.
24. Tessier PM, Lenhoff AM, Sandler SI. Rapid measurement of protein osmotic second virial coefficients by self-interaction chromatography. *Biophys J*. 2002; 82:1620–31. [PubMed: 11867474]
25. Valente JJ, Verma KS, Manning MC, Wilson WW, Henry CS. Second virial coefficient studies of cosolvent-induced protein self-interaction. *Biophys J*. 2005; 89:4211–8. [PubMed: 16199499]
26. Ahamed T, Ottens M, van Dedem GWK, van der Wielen LAM. Design of self-interaction chromatography as an analytical tool for predicting protein phase behavior. *J Chromatogr A*. 2005; 1089:111–124. [PubMed: 16130779]
27. Johnson HR, Lenhoff AM. Characterization and suitability of therapeutic antibody dense phases for subcutaneous delivery. *Mol Pharm*. 2013; 10:3582–3591. [PubMed: 24011376]
28. Collins KD. Ions from the Hofmeister series and osmolytes: effects on proteins in solution and in the crystallization process. *Methods*. 2004; 34:300–311. [PubMed: 15325648]
29. Lewus, RA. PhD thesis. University of Delaware; 2011. Monoclonal antibody interactions and phase behavior.
30. Dumetz AC, Snellinger-O'Brien AM, Kaler EW, Lenhoff AM. Patterns of protein – protein interactions in salt solutions and implications for protein crystallization. *Protein Sci*. 2007; 16:1867–1877. [PubMed: 17766383]

31. Zhang F, Roosen-Runge F, Skoda MWA, Jacobs RMJ, Wolf M, Callow P, Frielinghaus H, Pipich V, Prévost S, Schreiber F. Hydration and interactions in protein solutions containing concentrated electrolytes studied by small-angle scattering. *Phys Chem Chem Phys*. 2012; 14:2483–93. [PubMed: 22249363]
32. Lilyestrom WG, Shire SJ, Scherer TM. Influence of the cosolute environment on IgG solution structure analyzed by small-angle X-ray scattering. *J Phys Chem B*. 2012; 116:9611–9618. [PubMed: 22827493]
33. Muschol M, Rosenberger F. Liquid–liquid phase separation in supersaturated lysozyme solutions and associated precipitate formation/crystallization. *J Chem Phys*. 1997; 107:1953.
34. Scherer TM, Liu J, Shire SJ, Minton AP. Intermolecular interactions of IgG1 monoclonal antibodies at high concentrations characterized by light scattering. *J Phys Chem B*. 2010; 114:12948–12957. [PubMed: 20849134]
35. George A, Wilson WW. Predicting protein crystallization from a dilute solution property. *Acta Crystallogr D Biol Crystallogr*. 1994; 50:361–365. [PubMed: 15299385]
36. Bonneté F, Vivarès D. Interest of the normalized second virial coefficient and interaction potentials for crystallizing large macromolecules. *Acta Crystallogr Sect D Biol Crystallogr*. 2002; 58:1571–1575. [PubMed: 12351864]
37. Ebel C, Faou P, Zaccai G. Protein–solvent and weak protein–protein interactions in halophilic malate dehydrogenase. *J Cryst Growth*. 1999; 196:395–402.
38. Hitscherich C, Kaplan J, Allaman M, Wiencek J, Loll PJ. Static light scattering studies of OmpF porin: implications for integral membrane protein crystallization. *Protein Sci*. 2000; 9:1559–1566. [PubMed: 10975577]
39. Neal BL, Lenhoff AM. Excluded volume contribution to the osmotic second virial coefficient for proteins. *AIChE J*. 1996; 41:1010–1014.
40. Connolly M. The molecular surface package. *J Mol Graph*. 1993; 11:139–141. [PubMed: 8347567]
41. Janin J, Miller S, Chothia C. Surface, subunit interfaces and interior of oligomeric proteins. *J Mol Biol*. 1988; 204:155–164. [PubMed: 3216390]

Figure 1. Instantaneous and equilibrium phase boundaries for Mab A in ammonium and lithium sulfate solutions at pH 7.0 and 5.0.

Figure 2.

The phase diagram for Mab A in sodium chloride solutions at pH 7.0. All solution conditions tested resulted in phase separation.

Figure 3.

Instantaneous phase boundaries of Mab A in ammonium sulfate, lithium sulfate, and sodium chloride solutions with varying amounts of PEG 3350. The trends are consistent with depletion effects, except in low PEG concentrations in $(\text{NH}_4)_2\text{SO}_4$, where little effect is seen.

Figure 4.

Osmotic second virial coefficient of Mab B in ammonium and lithium sulfate solutions at pH 7.0 and 5.0. The value used for the hard sphere contribution to B_{22} , that is the value when $W=0$ in eqn. (1), was 0.19×10^{-4} mol ml/g².

Figure 5.

Instantaneous and equilibrium (two month) phase boundaries for Mab B in ammonium and lithium sulfate solutions at pH 7.0 and 5.0. The order of the instantaneous boundaries is in agreement with the interaction data, but that of the long-time boundaries is not.

Figure 6.

Instantaneous phase boundaries for Mab B in ammonium and lithium sulfate solutions, pH 7.0, with PEG 3350. A low polymer concentration results in increased repulsion, but a further increase in polymer concentration gives rise to effects consistent with those expected from depletion interactions.

Figure 7. B_{22} values in sodium chloride solutions at pH 7.0. Five antibodies are compared. Mab A and Milli004 are the only antibodies that displayed negative B_{22} values at low sodium chloride concentrations.

Figure 8.

B_{22} values and instantaneous phase boundaries in ammonium and lithium sulfate solutions at pH 7.0. Eight antibodies are compared; B_{22} values (open symbols) and phase boundaries (closed symbols) correlate well in lithium sulfate solutions but less strongly in ammonium sulfate solutions. The value used for the hard sphere contribution to B_{22} was 0.19×10^{-4} mol mL/g².

Figure 9. Instantaneous phase boundaries of antibodies in constant concentrations of ammonium sulfate and varying pH.

Figure 10.

B_{22} values of solutions that yield apparent crystals (including fuzz balls) are displayed along with George and Wilson's crystallization slot³⁰ (blue) and a range based on the dimensionless slot³⁶ (dashed lines). The arrows indicate that crystallization occurs at salt concentrations beyond those where interactions could be measured. Many of the values for which crystallization occurs fall within the slot, but a significant number are outside of the slot. The B_{HS} value is $0.19 \times 10^{-4} \text{ mol mL/g}^2$.