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Evaluation of effects of pH and ionic strength on colloidal stability of IgG solutions by PEG-induced liquid-liquid phase separation

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Colloidal stability of IgG antibody solutions is important for pharmaceutical and medicinal applications. Solution pH and ionic strength are two key factors that affect the colloidal stability of protein solutions. In this work, we use a method based on the PEG-induced liquid-liquid phase separation to examine the effects of pH and ionic strength on the colloidal stability of IgG solutions. We found that at high ionic strength (≥0.25M), the colloidal stability of most of our IgGs is insensitive to pH, and at low ionic strength (≤0.15M), all IgG solutions are much more stable at pH 5 than at pH 7. In addition, the PEG-depletion induced force is less efficient in causing phase separation at pH 5 than at pH 7. In contrast to the native inter-protein interaction of IgGs, the effect of depletion force on phase separation of the antibody solutions is insensitive to ionic strength. Our results suggest that the long-range electrostatic inter-protein repulsion at low ionic strength stabilizes the IgG solutions at low pH. At high ionic strength, the short-range electrostatic interactions do not make a significant contribution to the colloidal stability for most IgGs with a few exceptions. The weaker effect of depletion force at lower pH indicates a reduction of protein concentration in the condensed phase. This work advances our basic understanding of the colloidal stability of IgG solutions and also introduces a practical approach to measuring protein colloidal stability under various solution conditions. Published by AIP Publishing. [http://dx.doi.org/10.1063/1.4966708]

I. INTRODUCTION

Antibodies, especially the IgG type, are an important class of proteins. They play a central part in the antibody-mediated immune system of human body.1 Pharmaceutical IgGs have become a major category of protein drugs for the treatment of various diseases such as cancers and autoimmune disorders.2 Both antibody repertoire of human body and the antibody drug candidates in portfolio of pharmaceutical companies contain a large number of different antibody molecules. All IgG molecules have roughly the same size and the Y-like overall shape as shown in Fig. 1. The IgG molecules of the same subclass also share large parts of their amino acid sequence, but differ in the complementarity-determining regions (CDRs) in the Fab domains (Fig. 1). The variations of amino acid residues of CDRs could lead to a diversity in the inter-protein interactions. When these interactions are attractive, one can expect the fully folded protein molecules to self-associate into protein condensed phases.

Indeed, numerous studies reported that IgG solutions undergo various condensation phenomena including crystallization, liquid-liquid phase separation, aggregation, and gelation.3–13 These protein condensations involve folded globular protein molecules and thus are different manifestations of the colloidal instability of protein solutions. In vivo condensation of natural IgGs underlies the cryoglobulinemia complication in some blood cancers.10 Pharmaceutical IgGs with low colloidal stability are also a concern for antibody drug development. Therefore, evaluation of colloidal stability of IgG solutions is important for medical and pharmaceutical applications. In addition, the universal Y-like shape of the IgG molecules and the diversity of the inter-protein interactions make them an interesting system for theoretical study of the phase behavior of non-spherical colloidal particles.

The colloidal stability of an IgG solution is reflected by its propensity to undergo crystallization, liquid-liquid phase separation, and colloidal aggregation. However, studies of crystallization and aggregation are not always suitable for evaluating colloidal stability of protein solutions. First, crystallization and aggregation are strongly controlled by kinetic factors that depend on specific experimental conditions and are often difficult to predict. Also, IgGs, as large and flexible protein molecules, are generally difficult to crystallize. Aggregation is not associated with a well-defined
quantity, like solubility, that can be used as a measure of colloidal stability. In contrast, liquid-liquid phase separation is a phase transition that occurs at well-defined solution conditions. Liquid-liquid phase separation is not kinetically limited because nucleation of the liquid condensed phase is usually fast and, at high degree of supersaturation, the spinodal decomposition spontaneously occurs without nucleation. Moreover, liquid-liquid phase separation in protein solutions is thermodynamically metastable with respect to crystallization and aggregation.7,12,14,15 From a thermodynamic perspective, when liquid-liquid phase separation occurs, the kinetically hindered crystallization and aggregation will eventually occur given that there is sufficient time. In other words, liquid-liquid phase separation marks a higher limit of the chemical potential for a protein in crystals and colloidal aggregates. Therefore, the very nature of liquid-liquid phase separation makes it useful for evaluating colloidal stability of IgG solutions.

One practical challenge is that liquid-liquid phase separation is rarely observed in IgG solutions. For most IgG solutions, the inter-protein attractive interactions are so weak that liquid-liquid phase separation does not occur at temperatures above the freezing point of an aqueous solution. One method to overcome this obstacle is to add a nonionic polymer, polyethylene glycol (PEG), into the IgG solutions. PEG molecules in solution are sterically excluded from the volume occupied by protein molecules. This exclusion effect creates additional inter-protein attraction called depletion force,16–20 which can cause protein phase separation above the freezing point. Importantly, PEG normally does not interfere with the native inter-protein interactions, since PEG molecules are excluded from the interaction areas of protein molecules. In practical applications, PEG is commonly used as a protein precipitant to promote protein precipitation or crystallization. In previous studies, we have demonstrated that PEG can be used to induce liquid-liquid phase separation universally in IgG solutions.12 We also developed a method based on the PEG-induced liquid-liquid phase separation to evaluate the colloidal stability of different IgGs at a physiological pH.11

Colloidal stability of a protein solution is determined not only by the properties of the protein molecule but also by the solution condition. Two important factors of the solution condition are pH and ionic strength. The solution pH affects the charge state of the protein surface21 and thereby modulates the magnitude, polarity, and spatial distribution of the electrostatic interactions between protein molecules. On the other hand, ionic strength regulates the range and the magnitude of electrostatic interactions by the ion screening effect.22 As it is the case for regular colloids, pH and ionic strength could markedly affect the stability of protein solutions. Therefore, in this work, we examined the effects of pH and ionic strength on colloidal stability of IgG solutions by investigating the PEG-induced liquid-liquid phase separation. The pH and ionic strength effects on colloidal stability can also be estimated by measuring the zeta potentials of proteins. However, our method provides two unique advantages over zeta potential measurements: first, the propensity to undergo liquid-liquid phase separation directly characterizes the colloidal stability of protein solutions, while zeta potential measurements only capture the net surface charge of a protein molecule which usually does not account for all inter-protein interactions in the condensed phase; second, the PEG-induced liquid-liquid phase separation experiments are easier to perform as they do not require any training or specialized equipment. Another way to study colloidal stability of protein solutions is to determine virial coefficients by scattering techniques, including light scattering, X-ray scattering, and neutron scattering.23–26 Most scattering experiments measure pair-wise interactions between colloidal particles, i.e., the second virial coefficient. On contrary, the PEG-induced phase separation experiments measure the integrated overall interactions in the condensed phase. In this regard, the phase separation method is complementary to the scattering methods. When the scattering instruments are not available, the PEG-induced phase separation provides an alternative method for rapidly quantifying colloidal stability of protein solutions.

II. MATERIALS AND METHODS

A. Materials

Fully human monoclonal IgG antibodies mAb1, mAb2, mAb3, mAb4, mAb5, mAb6, mAb7, and mAb8 were produced at Amgen, Inc. All antibodies were in the monomeric form with purity higher than 95%. mAb1-mAb4 belong to the human IgG2 subclass. mAb5-mAb8 belong to the human IgG1 subclass. The isoelectric points of mAb1-mAb8 determined by capillary isoelectric focusing were respectively 7.2, 8.8, 6.8, 7.8, 9.0, 8.7, 8.9, and 7.5 (the cIEF data not shown). The acetate buffers of different concentrations (0.02M, 0.15M, 0.25M, 0.3M, and 0.5M) were prepared using sodium acetate salt and glacial acetic acid. The 0.02M phosphate buffer was prepared with Na2HPO4 and NaH2PO4 salts. Polyethylene glycol with the molecular weight 8000 Da and purity greater than 98% was prepared in 0.02M phosphate buffer.

FIG. 1. Crystallographic structure of an IgG molecule (DOI: 10.2210/pdb1IGT/pdb in the Protein Data Bank). The graph is rendered using VMD 1.9.1. Two identical heavy chains are shown in red, and two identical light chains are in blue. The three globular domains are two identical “antigen-binding domains” (Fab) and a “crystallizable domain” (Fc). The complementarity-determining regions (CDRs) represent the hyper-variable parts of IgG.
than 99% (PEG8000, Sigma-Aldrich, St. Louis, MO) was used.

**B. Solubility measurement of PEG-induced liquid-liquid phase separation**

Stock solutions of 2 mg/ml and 4 mg/ml IgG were prepared at a desired pH and ionic strength. Stock solutions of PEG8000 were prepared at a 2 × higher concentration than the final PEG concentration. Note that the highly concentrated PEG increased the solution pH, especially at pH 5.2 and 5.4. Thus, it was necessary to adjust solutions to the desired pH with glacial acetic acid following dissolution of PEG in the acetate buffer. Subsequently, equal volumes of protein and PEG stock solution were mixed at a room temperature. When the initially transparent samples were brought to 4 °C, they quickly became cloudy. The samples were then incubated at 4 °C for 24 h to achieve equilibrium. After incubation, the white protein precipitate sedimented to the bottom of the test tubes. Each sample was briefly centrifuged at 4 °C before an aliquot of the supernatant was carefully removed to determine the protein concentration. Supernatant protein concentrations were measured by a precalibrated 1100 Series HPLC system (Agilent Technologies, Santa Clara, CA) using a ProPac WCX-10 analytical column (weak cation-exchange, 4 mm × 250 mm; Dionex, Sunnyvale, CA) and a UV detector. Repeated measurements showed that the protein concentration in the supernatant did not change after 24 and 48 h of incubation. Therefore, the equilibrium protein concentration after a one-day incubation was taken as the solubility of the IgG solutions. We also found that the solubility of an IgG under the same solution condition did not depend on the initial protein concentration between 1 and 2 mg/ml.

**C. Measurement of phase separation temperature of PEG-induced liquid-liquid phase separation**

The phase separation temperatures of the IgG-PEG mixture solutions were determined by the turbidity measurement. The sample in a test tube was placed in a thermostated light-scattering stage. A laser beam (He-Ne 4 mW, 633 nm) was directed through the sample, and the intensity of transmitted light was measured by a photodiode and recorded by a power meter (1936-C, Newport). The temperature of the sample was then lowered from a room temperature by 0.2 °C every 5 min. At a temperature, T_{cloud}, the sample became cloudy and the transmitted intensity rapidly dropped. This clouding marked the onset of phase separation. After clouding occurred, the temperature was raised and the sample became clear at another temperature, T_{clear}. The average of T_{cloud} and T_{clear} was taken as the estimate of the liquid-liquid phase separation temperature.

**III. RESULTS AND DISCUSSION**

**A. PEG-induced liquid-liquid phase separation in IgG solutions**

Upon addition of PEG we observed precipitation in our IgG solutions at a constant temperature (Fig. 2(a)). The resulting protein concentrations in the supernatants were significantly lower than those in the original solutions. Thus, the observed white precipitates were a protein-rich phase. Under the light microscope, these precipitates were found to be liquid droplets that coalesced when in contact (Fig. 2(b)). The precipitates completely dissolved when the solution temperature was increased. The observed features of this thermally reversible protein condensation were consistent with a typical liquid-liquid phase separation process. An alternative way to observe liquid-liquid phase separation is to lower the temperature of a homogenous IgG-PEG solution. When the temperature falls below a well-defined clouding temperature, the condensation occurs. When the temperature is subsequently increased, the solution clears again. For all of our IgGs, both clouding and clearing events occurred within just a few seconds. In contrast to the nucleation-dependent and relatively slow crystallization and aggregation, the fast kinetics of clouding and clearing of our samples was consistent with liquid-liquid phase separation.

In previous studies, we have demonstrated that liquid-liquid phase separation can be universally induced in IgG solutions at pH 7 by addition of PEG. Since most IgGs have basic isoelectric points (pI from 6.5 to 10), pharmaceutical IgGs are often formulated at a moderately low pH (pH 5-6) and ionic strength (<0.15M) for high colloidal stability. The colloidal stability is mostly due to the net repulsive electrostatic interactions between protein molecules. Indeed, we found that most IgG solutions in 20 mM acetate at pH 5.2 remain transparent even in the presence of high concentrations of PEG (up to 20% w/w PEG8000). To observe phase separation, we had to increase the concentration of buffer salts up to 0.5M. At the high ionic strength, the range and the strength of electrostatic interactions were reduced due to the screening effect of the counter-ions. Thereby, we were able to observe the PEG-induced liquid-liquid phase separation in all of our IgG solutions over the pH range from 5.2 to 7.2 at the ionic strength of 0.5M.
In our experiments, we used sodium phosphate buffers for solutions at pH 7.2, 6.6, and 6.0 and sodium acetate buffers for solutions at pH 5.4 and pH 5.2. The ionic strength of both buffers was 0.5 M. To examine the effect of the type of buffer salt on phase separation, we measured the clouding and clearing temperatures of the IgG-PEG mixture solutions in 0.5 M acetate and phosphate buffers at pH 6.0. The phase separation temperatures in the two different buffers were very similar (the difference was within 0.5°C, data not shown). Therefore, we did not consider the effect of the type of buffer salts on phase separation hereafter.

B. The pH effect on the PEG-induced liquid-liquid phase separation in IgG solutions

In our experiments, higher pHs favor the PEG-induced liquid-liquid phase separation. Upon PEG-induced liquid-liquid phase separation, the protein concentration in the protein-poor phase (the supernatant) drops to the equilibrium value after isothermal incubation. After incubation at 4°C, we measured the equilibrium IgG concentration in supernatants for samples with different PEG concentrations (Fig. 3). Fig. 3 shows that, at a given pH, the equilibrium protein concentration decreases as the PEG concentration increases.

FIG. 3. The IgG solubility via liquid-liquid phase separation as a function of PEG concentration and pH at 4°C.
This result is expected since the depletion interaction (the additional inter-protein attraction) introduced by PEG increases with PEG concentration. With the exception of mAb8, the equilibrium protein concentration in Fig. 3 decreases as pH increases. This result suggests that it is more difficult to induce phase separation at low pH using PEG.

The lower equilibrium protein concentration at higher pH indicates a stronger overall inter-protein attraction, which consists of native inter-protein interactions and the PEG-induced depletion force. It is important to note that the equilibrium concentrations of IgGs in our experiments are independent of the protein concentration of the initial solution. From a theoretical perspective, this observation can only be expected for phase separation in dilute protein solutions. In all our experiments, the initial IgG solutions are quite dilute (<2 mg/ml). When phase separation occurs in a dilute IgG solution, the protein concentration in the condensed phase is generally very high (hundreds of mg/ml).

Due to the steric exclusion of the closely packed protein molecules, the condensed phase is essentially incompressible and the PEG molecules only stay in the protein-poor phase. In this limiting case, the protein concentration in the supernatant has a well-defined value at a given temperature and PEG concentration. Hereafter, we will refer to the equilibrium IgG concentration in supernatant as the protein solubility under the given solution condition. The solubility measurements enable us to obtain information on the native and PEG-induced inter-protein interactions. In an extensive body of historic work, the PEG-induced inter-protein interaction has been studied both experimentally and theoretically. With only a few exceptions where specific PEG-protein interactions were seen, the PEG-induced inter-protein interaction is a purely entropic depletion force.

To capture the physical meaning of the pH-dependence of IgG solubility shown in Fig. 3, we have performed following theoretical analysis based on the assumptions of incompressible protein condensed phase and purely entropic depletion interaction. In a previous work, we have shown that, with these approximations, the IgG solubility measured in our experiment, $c_1$, as a function of the osmotic pressure produced by PEG, $\Pi_2$, can be expressed in a simple equation,

$$\ln \left( \frac{c_1}{c_0} \right) = \ln \left( \frac{M_1}{v_0N_Ac_0} \right) - \frac{\varepsilon_B}{kT} + (c_0 - c) \frac{\Pi_2}{kT},$$

where $c_0 \equiv 1 \text{ g/l}$, $N_A$ is the Avogadro’s number, $k$ is the Boltzmann constant, $T$ is the absolute temperature, and $M_1$ is the molecular weight of the protein (taken as 150 kg/mol). In this equation, there are three quantities that characterize the properties of the protein-polymer mixtures undergoing phase separation: $v$ is the volume excluded for PEG8000 (i.e., the volume inaccessible to the mass center of PEG molecules) by an IgG molecule in the dilute phase; $v_0$ is the excluded volume per IgG molecule in the condensed phase; and $\varepsilon_B$ is the binding energy of an IgG molecule in the condensed phase (Fig. 4). When PEG molecules are completely excluded from the condensed phase, the excluded volume $v_0$ is equal to the solution volume per IgG molecule. The binding energy, $\varepsilon_B$, is an important parameter that characterizes the strength of interactions between native protein molecules. The attractive inter-protein interactions that drive liquid-liquid phase separation could also result in other protein condensation phenomena such as colloidal aggregation and crystallization. In protein solutions, liquid-liquid phase separation is metastable with respect to crystallization and aggregation, i.e., the $\varepsilon_B$ mentioned for liquid-liquid phase separation is the lower limit of the binding energy in the solid condensed phases. Therefore, $\varepsilon_B$ is a measure of the colloidal stability of protein solutions in a general sense.

In Eq. (1), the bracketed term characterizes the native inter-protein interaction in the condensed phase: $\ln \left( \frac{M_1}{v_0N_Ac_0} \right)$ is the translational entropy of a protein molecule; the binding energy, $\varepsilon_B$, represents all other parts of free energy including energetic and entropic components. The second term on the right side of Eq. (1) describes the effect of depletion force induced by the addition of PEG. It is easy to see that $(c_0 - c) \Pi_2$ is the volumetric work done by the osmotic pressure of PEG in the process to move an IgG molecule from the dilute phase to the condensed phase. The osmotic pressure of PEG, $\Pi_2$, can be calculated from the weight fraction of PEG, $c_2$, using a semi-empirical equation of state.

$$\frac{\Pi_2}{N_AkT} = \frac{\rho}{M_2} c_2 \left[ 1 + 0.49 \left( \frac{c_2}{c_2^*} \right)^{5/4} \right].$$

Here $\rho \equiv 1 \text{ g/ml}$ is the density of the solution, and $M_2$ is the molecular weight of PEG, and $c_2^* \equiv \rho \sigma^{-0.8}/b_2$ is the dilute-semidilute crossover concentration of PEG, where $\sigma$ is the number of ethylene glycol units in a PEG molecule and $b_2 = 0.825 \text{ ml/g}$ is the partial specific volume of PEG.

Eq. (1) shows that pH could affect the protein solubility through altering the free energy of native inter-protein interactions and changing the work done by the PEG-induced interactions.
depletion force. Using the data in Fig. 3, we plotted $\ln\left(\frac{c_1}{c_0}\right)$ versus $H_2/NAkT$ as shown in Fig. 5. According to Eq. (1), one can fit the data in Fig. 5 using a linear function. The slope, $N_A(v_0 - v)$, and the intercept, $\ln\left(\frac{M_1}{v_0 c_0 kT}\right)$, of the linear fit respectively characterize the effect of depletion force and native inter-protein interactions on phase separation. As shown in Fig. 4, the excluded volume, $v$, in the dilute phase only depends on the geometry of IgG molecules and the size of PEG. Since all IgG molecules have similar shape and size, $v$ is approximately a constant for a given PEG. In a previous study,$^{11}$ we have analyzed the dependence of $v$ on the PEG molecular weight and estimated that $v \approx 387$ nm$^3$ for PEG8000. Knowing the value of $v$, the excluded volume in the condensed phase, $v_0$, can be deduced from the slope of the linear fits in Fig. 5, and the binding energy $\varepsilon_B$ can then be deduced from the intercept. We wrote the binding energy at a given pH as $\varepsilon_B = \varepsilon_{B0} + b\delta pH$, where $\varepsilon_{B0}$ is the binding energy at a reference pH, $\delta pH$ is the change of pH with respect to the reference pH, and $b$ is the proportionality coefficient. Using this expression of $\varepsilon_B$ and Eq. (1), we were able to simultaneously fit the data in each graph of Fig. 5 (the goodness of fit, $R^2 > 0.97$). The resulting values of $v_0$ and $\varepsilon_{B0}$ are summarized in Fig. 6. Alternatively, one can use Eq. (1) to fit the data individually without the constraint of $\varepsilon_B$ (Fig. S1 of the supplementary material). The results thus obtained are similar to Fig. 6.

In Fig. 6(a), for all of our IgGs, $v_0$ increases as pH decreases below 6. The pH dependence of $v_0$ suggests that the protein concentration in the condensed phase decreases when pH is lowered. Intuitively, one can expect a less dense condensed phase at a lower pH, since the electrostatic interactions between IgG molecules become less attractive.

FIG. 5. $\ln\left(\frac{c_1}{c_0}\right)$ as a function of the normalized PEG osmotic pressure $H_2/NAkT$. The plots are made based on the data in Fig. 3. The solid lines are the linear fits using Equation (1).
as pH shifts away from their isoelectric point. However, the lower density of condensed phase cannot be attributed to the overall repulsion between net surface charges on IgGs. In our experiments, the ionic strength was high (0.5 M) and the range of electrostatic interaction was short (Debye length is ∼7 nm). This length of ionic interactions is much shorter than the distance between the centers of IgG molecules in the condensed phase (√ε0 is ∼7 nm). Therefore, the increase of v0 at low pH is due to the changes of localized electrostatic interactions between the protein surfaces. Indeed, previous neutron scattering studies have shown that concentrated IgG solutions can have very different solution structures.26,30 For most of our IgGs, v0 is insensitive to pH changes above pH 6.0. This observation is in agreement with the fact that the pKa values of ionic groups of amino acid residues are mostly far from pH 7.

The pH-dependence of v0 has a strong effect on the work done by the PEG-induced depletion force. As shown in Eq. (1), the strength of the depletion force is associated with the difference between v and v0. The change in v0 is more significant with respect to v − v0 than that of v0 itself. Therefore, the slope in Fig. 5 is expected to be sensitive to the change in v0. In contrast, the ln(v0) term of the intercept is only marginally affected. Note that the condensed phase is still highly concentrated even at low pH. Direct measurement of the protein concentration in the condensed phase is impractical because the amount of the condensed phase is very small, and the contamination from the dilute bulk phase during the liquid handling is almost inevitable. Nevertheless, the v0 measured in our experiments can be used to estimate the protein concentration in the condensed phase: ε1/M = M1/NAb0. The largest v0 in Fig. 6 (∼330 nm) corresponds to the IgG concentration ∼750 mg/ml. Therefore, the condensed phase in all of our experiments is very crowded, which is consistent with our initial assumptions.

While the work of the depletion force in phase separation exhibits significant dependency on pH, we found that the magnitude of the overall native inter-protein interactions for the majority of our IgG is essentially independent of pH. With the exceptions of mAb7 and mAb8, the intercepts of the linear fits in Fig. 5 do not change with pH within the experimental error. Similarly, Fig. 6(b) shows that the binding energy, εB, of IgGs (mAb1-mAb6) in the condensed phase exhibits little pH dependence. This result suggests that the average inter-protein interaction of the IgGs in the condensed phase is insensitive to pH, even though the changes in v0 suggest that the less-dense configurations of protein molecules are favored at low pH. In contrast, the binding energies, εB, of mAb7 and mAb8 show strong pH dependency in opposite directions. Apparently, a stronger pH dependence of εB indicates that the proteins have more ionic groups with pKa close to the pH range of 5.2-7.2. On the other hand, the isoelectric points of mAb7 and mAb8 are within the pH range of other IgGs. Therefore, the pH dependence of εB of mAb7 and mAb8 demonstrates the importance of localized electrostatic interactions, instead of the net interactions signified by zeta potential, for colloidal stability of protein solutions at high ionic strengths. The IgGs with abnormal inter-protein interactions are likely to present challenges for biopharmaceutical development or even play a role in in vivo pathological condensation in diseases such as cryoglobulinemia.

C. The effect of ionic strength on the PEG-induced liquid-liquid phase separation in IgG solutions

Ionic strength of a solution is another important factor that controls the electrostatic interactions between protein molecules. We examined the pH dependence of v0 and εB for mAb1 at two lower ionic strengths (0.3 M and 0.25 M) by measuring the protein solubility in PEG-induced liquid-liquid phase separation. (Fig. 7) Interestingly, we found that v0 and εB did not strongly depend on ionic strength. Their variations were within the experimental error. The experiments on mAb2 (Fig. S2 of the supplementary material) showed similar results to mAb1. On the other hand, we already knew that the PEG-induced phase separation showed a very different behavior at ionic strengths lower than 0.15 M. At pH 5.2 and ionic strength 0.15 M, we could not induce liquid-liquid phase separation for any of our IgGs using up to 20% w/w PEG8000. At pH 7 and 0.15 M ionic strength, liquid-liquid phase separation

FIG. 6. The volume per IgG molecule v0 (a) and the binding energy of an IgG molecule εB (b) in the condensed phase as a function of pH. These values were obtained by fitting the data in Fig. 5 using Eq. (1). The uncertainties of v0 and εB associated with the fitting are respectively within 10 nm³ and 0.5 kJ/mol.
was observed in our IgG solutions by addition of as little as ~6% w/w PEG8000. These results suggested that the effect of ionic strength on the PEG-induced liquid-liquid phase separation can be divided into two distinct regions: (1) below 0.15M, the repulsion between surface charges of IgGs at low pH completely prevents phase separation, and the PEG-induced depletion force is not sufficient to overcome the repulsion; (2) above 0.25M, the repulsion is screened by the counter-ions, but further increases in the ionic strength impose only an insignificant effect on the overall inter-protein interaction. That is to say, there is a critical ionic strength between 0.15M and 0.25M. One plausible explanation for this sharp transition of the ionic strength effect is the following: below the critical ionic strength, the range of the electrostatic repulsion is longer than the range of the depletion force induced by PEG, which thereby creates a long-range energy barrier which cannot be overcome by increasing the strength of the short-range depletion force. Above the critical ionic strength, the electrostatic interactions are short-range with respect to the depletion force and are relatively weak compared to other types of short-range inter-

protein interactions. Thus, a further increase of ionic strength has only a marginal effect on the total inter-protein interaction. To elucidate this transition, we calculated the Debye-Hückel length of charge carriers in our solutions. The Debye length increases from 0.4 nm to 0.8 nm as the ionic strength of solution decreases from 0.50M to 0.15M. For comparison, the range of depletion interaction of PEG is approximately 0.5Rg,17,18 where Rg ≈ 0.0287M20.55 is the radius of gyration of PEG and M2 is the molecular weight of PEG. The range of depletion interaction for PEG8000 is about 1.3 nm. Assuming the effective range of electrostatic interaction is proportional to the Debye length, the electrostatic repulsion can outrange the depletion interaction as ionic strength decreases.

IV. CONCLUSION

We have previously demonstrated that liquid-liquid phase separation can be universally induced in IgG antibody solutions by the addition of PEG. Liquid-liquid phase separation provides a means to probe the attractive interactions between the native folded antibody molecules. In a previous study,11 we established a method based on the PEG-induced liquid-liquid phase separation at neutral pH to quantify the colloidal stability of different IgG antibodies, i.e., the propensity of the native proteins to undergo liquid-liquid phase separation as well as the closely related colloidal aggregation and crystallization. In this work, we demonstrated that this method can also be used at mildly acidic pH to compare the stability of different therapeutic IgGs which are typically formulated between pH 5 and 6. We also evaluated the effects of pH and ionic strength on the native inter-protein interactions of IgGs and the PEG-induced depletion force. We found that, at ionic strength above 0.25M, pH (from 5.2 to 7.2) has little effect on the strength of the native inter-protein interactions for most of our IgGs. However, the PEG-induced depletion force is less efficient in causing phase separation when pH is far away from an isoelectric point of the protein. At ionic strength lower than 0.15M, the PEG-induced liquid-liquid phase separation is prevented at acidic pH, but restored at high pH. The results of our experiments suggest that at a low ionic strength, the electrostatic repulsion between protonated IgGs at acidic pH has a longer range compared to the depletion force; at a high ionic strength, the short-range repulsion does not make significant contribution to the total interaction but it results in a decrease of protein concentration in the condensed phase. This work provides insights into the basic rules that govern colloidal stability of IgG antibodies under different solution conditions. This knowledge is valuable for practical applications such as formulation development of pharmaceutical antibodies. Our results also shed light on the effects of depletion interaction on phase transitions in solutions of highly charged proteins. Along the line of this study, future research on the interplay between the range of electrostatic repulsion (ionic strength) and the range of depletion interaction (depending on PEG molecular weight) could yield interesting results.
SUPPLEMENTARY MATERIAL

See supplementary material for Figures S1 and S2.

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