molecular pharmaceutics

Article

pubs.acs.org/molcularpharmaceutics

Quantitative Evaluation of Colloidal Stability of Antibody Solutions using PEG-Induced Liquid−Liquid Phase Separation

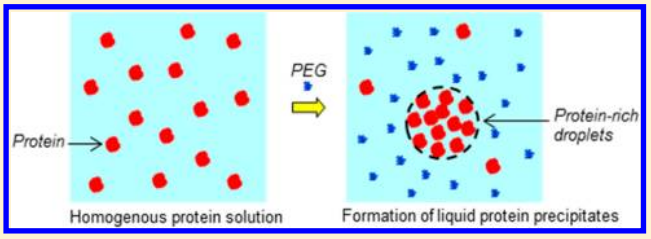
Ying Wang,[†,‡] Ramil F. Latypov,[‡,§] Aleksey Lomakin,[†] Julie A. Meyer,[§] Bruce A. Kerwin,[§] Suresh Vunnum,[§] and George B. Benedek*[,†,∥,⊥]

[†]Materials Processing Center, [∥]Department of Physics, and [⊥]Center for Materials Science and Engineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge, Massachusetts 02139, United States

[§]Process and Product Development, Amgen Inc., Seattle, Washington 98119, United States

Supporting Information

ABSTRACT: Colloidal stability of antibody solutions, i.e., the propensity of the folded protein to precipitate, is an important consideration in formulation development of therapeutic monoclonal antibodies. In a protein solution, different pathways including crystallization, colloidal aggregation, and liquid−liquid phase separation (LLPS) can lead to the formation of precipitates. The kinetics of crystallization and aggregation are often slow and vary from protein to protein. Due to the diverse mechanisms of these protein condensation processes, it is a challenge to develop a standardized test for an early evaluation of the colloidal stability of antibody solutions. LLPS would normally occur in antibody solutions at sufficiently low temperature, provided that it is not preempted by freezing of the solution. Poly(ethylene glycol) (PEG) can be used to induce LLPS at temperatures above the freezing point. Here, we propose a colloidal stability test based on inducing LLPS in antibody solutions and measuring the antibody concentration of the dilute phase. We demonstrate experimentally that such a PEG-induced LLPS test can be used to compare colloidal stability of different antibodies in different solution conditions and can be readily applied to high-throughput screening. We have derived an equation for the effects of PEG concentration and molecular weight on the results of the LLPS test. Finally, this equation defines a binding energy in the condensed phase, which can be determined in the PEG-induced LLPS test. This binding energy is a measure of attractive interactions between antibody molecules and can be used for quantitative characterization of the colloidal stability of antibody solutions.



KEYWORDS: *antibody, PEG, liquid−liquid phase separation, colloidal stability, binding energy*

1. INTRODUCTION

Therapeutic monoclonal antibodies are an increasingly important class of protein drugs being used for targets ranging from inflammation to cardiovascular disease and cancer.[1] During the various stages in the development of antibody drugs, multiple factors can affect their stability and result in various forms of protein condensation such as crystallization, aggregation, gelation, and liquid−liquid phase separation. Recently, a number of cases of such condensation of monoclonal antibodies have been reported.[2−8] In order to avoid these problems, it would be desirable to have an early stage solubility screening method to eliminate molecules that may present challenges in development. However, due to the diversity of antibody molecules and the various forms of condensation, standard solubility tests are not yet available in the biopharmaceutical industry. Current approaches for evaluating the solubility of protein therapeutics are based on salt (ammonium sulfate) induced precipitation[9,10] or PEG-induced protein precipitation.[11] The advantage of PEG-induced precipitation in comparison to the ammonium sulfate method is that the addition of salt can significantly alter the native interactions between protein molecules[12] while addition of PEG does not.[13] Here, we elucidate the mechanism of PEG-induced protein precipitation and put this method on a quantitative basis.

In this study, we consider condensation of normally folded IgGs caused by the attractive interaction between protein molecules. These condensations include crystallization, colloidal aggregation, gelation, and liquid−liquid phase separation. From a physicochemical perspective, the folded protein molecules can be viewed as colloidal particles, and their condensation signifies the loss of colloidal stability of the solution. Protein aggregation, often irreversible, can also be caused by unfolding of the protein molecules. The conformational stability of proteins associated with unfolding, which is outside the scope of this work, is usually tested at elevated temperature, at extreme pHs, or with mechanical agitations.[14,15]

Received: August 29, 2013
Revised: January 30, 2014
Accepted: March 27, 2014
Published: March 27, 2014

Here, we focus on the colloidal stability, which can be tested directly under storage or production solution conditions, without promoting chemical modifications and unfolding of the protein molecules. Protein molecules in their native folded conformation can attract each other through various types of interactions such as electrostatic interactions between surface charges, hydrophobic interactions, and formation of hydrogen bonds and salt bridges. The propensity to undergo colloidal condensation is determined by the overall effect of all these interactions.

Among the various types of colloidal condensations, liquid−liquid phase separation (LLPS) is of particular importance for the evaluation of the colloidal stability of protein solutions. LLPS is a spontaneous segregation of a homogeneous protein solution, below a certain temperature (LLPS temperature), into coexisting protein-dilute and protein-rich liquid phases.[16−24] LLPS in several different monoclonal antibody solutions have recently been reported.[2−8] In equilibrium, the chemical potentials of colloidal particles (protein molecules) in dilute and condensed phases are equal. Since, in the condensed phase, the spatial position of each particle is restricted by its neighbors, the chemical potential in the condensed phase typically has a lesser entropic component, and a larger "binding" energy component associated with neighbor-to-neighbor attractions. Thus, a lower temperature generally favors the condensed phases. The ranges of interprotein attractions are relatively small in comparison to the size of the protein molecules. As a result, the protein in the condensed and unordered liquid phase is "caged" by its neighbors to nearly the same degree as in the solid state, but has fewer neighbors and thus has a smaller binding energy than in the solid state. Therefore, the solid state of colloid particles with short-range attractions has a lower chemical potential than the condensed liquid phase. That means that the coexisting protein-rich and protein-dilute liquid phases are metastable with respect to the protein solid phases. This is well established both theoretically and experimentally for spherical colloids[25,26] and for quasispherical globular proteins exhibiting LLPS.[27,28] Recent experimental studies have confirmed that this is also true for Y-shaped IgG molecules.[7,29] In other words, if LLPS is observed in an IgG solution, crystallization and colloidal aggregation can also occur under the same solution conditions. That is why in protein solutions LLPS can only be observed when, for kinetic reasons, crystallization or aggregation occurs slowly after a significant lag time required for nucleation.

Observation of LLPS is an indication of the strength of the averaged overall interprotein attractive interactions which can also drive crystallization and colloidal aggregation.[4,5,8,19,30,31] As explained above, these other condensates are in principle more stable than coexisting liquid phases. Crystallization or colloidal aggregation is therefore possible, but might take a long time, in a range of conditions such as temperature, pH, ionic strength, and adjuvant concentrations around conditions conducive to LLPS. Thus, LLPS provides a universal tool for mapping the colloidal stability of antibody solutions. Furthermore, antibodies can also lose their solubility over time due to the accumulation of chemical modifications such as oxidation and deamidation.[32,33] These chemical modifications could cause an increase of the attractive interprotein interactions with or without (partial) unfolding, and eventually lead to condensation. The LLPS temperature is a sensitive measure of the collective attractions between proteins, and it may change in the presence of modified proteins.[34,35] Therefore, LLPS temperature can also serve as a useful indicator of the colloidal stability of the antibody solution over long-term storage.

Despite the great potential of LLPS in evaluating colloidal stability of therapeutic antibodies, the main constraint for its application is the absence of LLPS in most antibody solutions. While LLPS in some antibody solutions has been reported,[2−8] in most antibody solutions the overall attractive interprotein interactions are too weak to cause LLPS at temperatures above the freezing point of the solution.[29] In this work, we demonstrate that poly(ethylene glycol) (PEG), a common precipitant, can be used to induce LLPS in all antibody solutions at temperatures above the freezing point of the solution. With few exceptions, PEG is a "nonabsorbing" polymer for proteins.[13,36−39] Since PEG is excluded from the contact area of the interacting proteins, the addition of PEG alters neither the direct interprotein interaction nor the interactions between proteins mediated by other small molecules. Therefore, PEG-induced LLPS is a suitable method to assess the colloidal stability of pharmaceutically relevant antibody solutions. This method can also provide a basis for systematic studies of the effects of various excipients on the interprotein interactions and the colloidal stability of antibodies. Such studies of PEG-induced LLPS may help make judicious decisions during protein formulation development and thus reduce time and resources needed for drug development.

In this work, we demonstrate the utility of PEG-induced LLPS by comparing the colloidal stability of different monoclonal antibodies under different solution conditions. In section 3.1, we first show that the precipitation of IgGs can be induced by the addition of PEG, and the protein concentration in the supernatant over the precipitates (referred hereafter as the solubility) is a characteristic of each IgG under a given solution condition. Then, in section 3.2, we demonstrate that the PEG-induced precipitation is indeed liquid−liquid phase separation in IgG solutions. In the following section (3.3), we present examples of the utility of PEG-induced LLPS measurements for comparing colloidal stability of IgGs under various solution conditions. In section 3.4, we compare the ability of PEGs with different molecular weights to induce LLPS in IgG solutions. In the section 4.1, we explain the rationale for using LLPS to evaluate the colloidal stability of IgG solutions. In this section, we also discuss the technical pros and cons of the solubility method in comparison with two other alternative methods for characterizing PEG-induced LLPS. In addition, we point out several considerations to be kept in mind when performing the solubility measurement. In section 4.2, we present an equation for describing the solubility reduction in dilute IgG solutions as a function of PEG concentration. This equation involves a phenomenological parameter, $\Delta\nu$, which describes the increase of the solvent volume accessible to PEG upon one protein molecule going from dilute into condensed phase and serves to quantify the depletion interaction caused by PEG. By fitting our experimental data using this equation, we have calculated the parameter $\Delta\nu$ for PEG with a molecular weight 3350 Da. With the additional data for PEGs with molecular weights of 1500, 4600, 6000, and 8000 Da, we have also determined the dependence of $\Delta\nu$ on PEG molecular weight. With the value of $\Delta\nu$ known, our equations allow a prediction of reduction of IgG solubility upon LLPS at a given PEG concentration and molecular weight. Finally, we introduce the "binding energy" (ε_B) in the condensed phase, which can be deduced from the solubility measurement, as the characteristic

quantity representing the strength of attractive interactions between antibodies. The experimental procedure for determination of ε_B can be readily automated and optimized for high-throughput applications. In section 4.3, we discuss how the binding energy ε_B can be used for quantifying the colloidal stability of IgG solutions.

2. EXPERIMENTAL SECTION

2.1. Materials. Fully human monoclonal antibodies (denoted as mAb 1, mAb 2, mAb 3, mAb 4, and mAb 5) with purity greater than 95% were produced at Amgen Inc. All these antibodies belong to the human IgG2 subclass. The proteins were exhaustively dialyzed into the buffer used in the experiments. The 10× PBS (phosphate buffered saline) was purchased from Lonza (AccuGENE, Cat. # 51226, 0.017 M KH_2PO_4, 0.05 M Na_2HPO_4, 1.5 M NaCl, pH 7.4) and diluted ten times. The acetate buffer at pH near 5 was prepared from high purity reagents and sterile filtered prior to use. Cell broth (pH 6.9) was obtained from an Amgen CHO (Chinese hamster ovary) cell culture process and clarified by centrifugation.

2.2. Solubility Measurement. PEG-induced precipitation of the IgGs was investigated at 0 and 4 °C in the presence of 7−10% PEG-3350 in PBS at pH 7.2. In order to minimize nonequilibrium precipitation, sample preparation consisted of mixing 2× protein and 2× PEG solutions at a 1:1 volume ratio. After mixing, samples were preincubated at 37 °C for a few hours to redissolve nonequilibrium aggregates. Then, samples were placed in a water bath (Ecoline RE 106, Lauda Brinkmann) at either 0 or 4 °C and incubated for at least 24 h to reach equilibrium. The length of incubation was established by testing samples with different starting protein concentrations (1 and 2.5 mg/mL). After incubation, white precipitates were observed at the bottom of the test tubes and the supernatants were transparent. The samples were then centrifuged for 30 s in a centrifuge chilled to the incubation temperature. Aliquots of the supernatants (i.e., the dilute phase) were immediately carefully removed. All the above procedures we carried out in a cold room (4 °C). Thus, obtained samples of the dilute phase were analyzed by cation exchange chromatography (CEX). The CEX method was run on an Agilent 1100 Series HPLC system using a ProPac WCX-10 analytical column (weak cation exchange, 4 × 250 mm; Dionex, Sunnyvale, CA) at ambient temperature and at a flow rate of 0.8 mL/min.[40] The column was equilibrated with buffer A (20 mM acetate, 0.0025% sodium azide, pH 5.2), and the protein was eluted with a linear gradient of buffer B (20 mM acetate, 1 M sodium chloride, 0.0025% sodium azide, pH 5.2) from 0 to 33% over 30 min. Following elution, the column was washed with 100% buffer B for 2 min and re-equilibrated with buffer A for 5.5 min. Data were analyzed using Dionex Chromeleon software and the 280 nm signal was integrated to estimate protein peak area. The protein concentration was calculated on the basis of the chromatographic peak area relative to a control sample with known concentration. It is worth noting that the protein concentration can also be directly measured using UV spectrometry in the solubility assay of pure protein solutions. The absence of contamination from the precipitates was assured by measuring the protein concentration three times for the aliquots separately pipetted from each centrifuged sample. We will call the measured protein concentration in the supernatant "solubility" in the PEG-induced precipitation experiments.

2.3. Turbidity Measurement. In the turbidity method, a test tube containing the sample was placed in a thermostated light-scattering stage, whose temperature was initially set to be 37 °C, at which the solution remained homogeneous. A laser beam (He−Ne 4 mW, 633 nm) was directed through the sample, and the transmitted intensity of light was detected by a photodiode and registered to a power meter (1936-C, Newport). The temperature of the sample was then lowered by 0.2 °C every 5 min. At a particular temperature, T_{cloud}, the sample became visibly cloudy and the transmitted intensity rapidly dropped to below one tenth of the initial intensity. This clouding marks the onset of phase separation and is due to the formation of small droplets of a protein-rich phase in a dilute solution. Upon clouding, the temperature was then raised and the sample became clear again. The temperature at which clarification occurs is denoted by T_{clear}. The average of T_{cloud} and T_{clear} was taken as the LLPS temperature T_τ.

2.4. Quasielastic Light Scattering. All protein samples were filtered through a 0.1 μm Millipore filter and placed in a test tube. QLS experiments were performed on a light-scattering apparatus using a PD2000DLSPLUS correlator (Precision Detectors) and a Coherent He−Ne laser (35 mW, 632.8 nm; Coherent Radiation). The measurements were performed at a scattering angle of 90°. The measured correlation functions were analyzed by the Precision Deconvolve 5.5 software (Precision Detectors). The correlation functions were used to calculate the apparent diffusion coefficients, D, of proteins in solutions.

3. RESULTS

3.1. Precipitation of IgG Induced by Addition of PEG. For all the IgGs (mAb 1, mAb 2, mAb 3, mAb 4, and mAb 5) in our study, protein precipitation was observed upon addition of a sufficient amount of PEG into the protein solution (e.g., Figure 1A,B). Without PEG, none of these IgGs exhibited precipitation at the same concentration and temperature for over a week. During PEG-induced precipitation, IgGs in the supernatant over the precipitates remained in their monomeric form (e.g., Figure 2). The IgG concentration in the supernatant dropped and eventually reached an equilibrium value after incubating at a constant temperature (0 or 4 °C) for 24 h. The equilibrium concentration of each IgG in the supernatant depends on the incubation temperature and the amount of PEG added (Figure 3). At the same temperature and PEG concentration, the same equilibrium IgG concentration was reached for the samples with different initial protein concentrations. We can define this equilibrium concentration as the "solubility" of the IgG under the specific solution conditions.

As is shown in Figure 3, the solubility of IgGs decreases as the PEG concentration increases, which is caused by the attractive "depletion" interprotein interactions introduced by PEG.[13,36−39] Also, the solubility of IgGs increases with temperature (Figure 3), i.e., the precipitate dissolves upon increasing the solution temperature. At a fixed temperature and PEG concentration, the solubilities of the IgGs are different from one another and increase in the order of mAb 3, mAb 1, mAb 2, mAb 5, and mAb 4.

3.2. PEG-Induced Precipitation and Liquid−Liquid Phase Separation. To elucidate the nature of the precipitation observed in the solubility measurements above, we studied in more detail the phase behavior of two of the IgGs: mAb 1 and mAb 2. We found that the condensation of

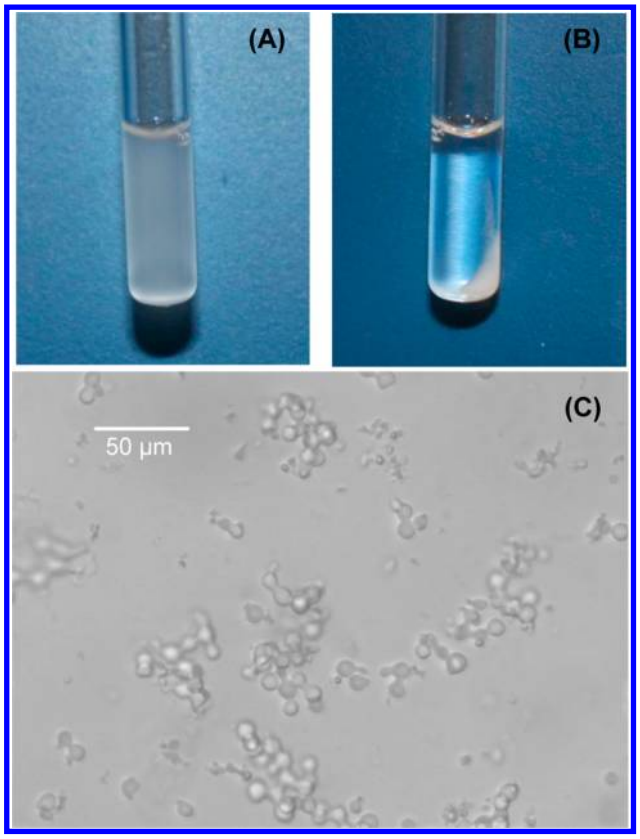


Figure 1. IgG precipitates produced by the addition of PEG. The initial solution of 1 mg/mL mAb 1 and 10% (w/w) PEG3350 was incubated at 21 °C. (A) The cloudy sample after 1 h incubation. (B) The sample after overnight incubation and 30 s centrifugation at 2000*g* at 21 °C. (C) The picture was taken with bright field microscope at 21 °C after 1 h incubation.

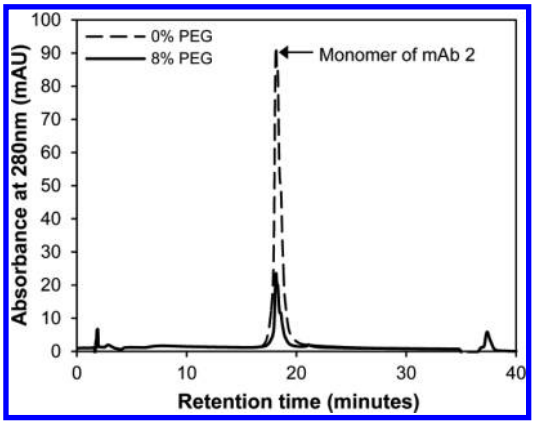


Figure 2. A CEX HPLC chromatogram of mAb 2 in the supernatant after incubating a solution with 1 mg/mL mAb 2 and 8% (w/w) PEG3350 at 0 °C for 3 days. A 1 mg/mL mAb 2 solution without PEG after the same incubation is used as the control sample.

these IgGs in the presence of PEG is reversible and the precipitates can be dissolved by increasing the solution temperature. Under the light microscope, the morphology of the IgG precipitates in our study appeared to be droplets (Figure 1C).

We conducted turbidity measurements to determine the temperature, T_τ, at which the phase transition takes place at various PEG concentrations. The results are shown in Figure 4.

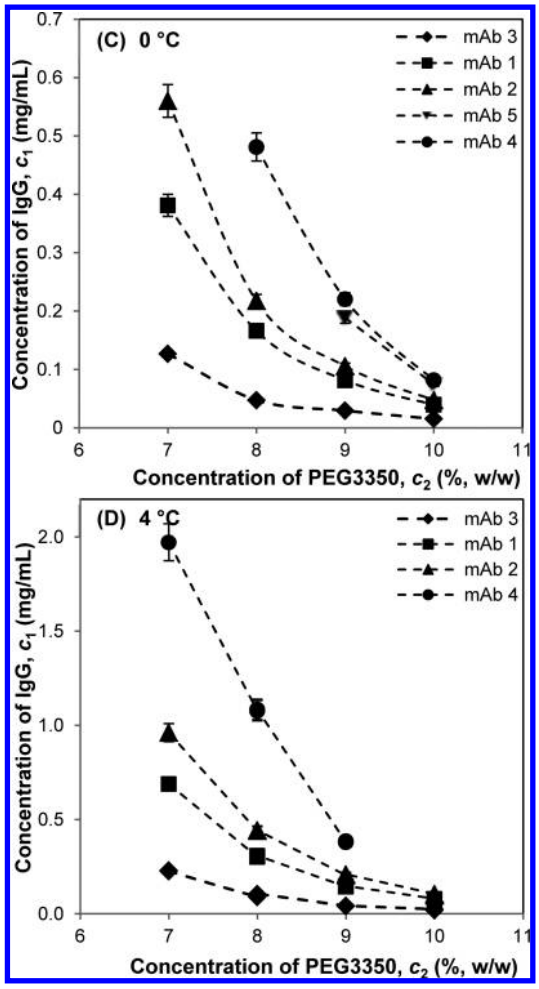


Figure 3. Equilibrium concentration of various IgGs in the supernatant as a function of the initial PEG concentration after incubation at (A) 0 °C and (B) 4 °C.

The temperature hysteresis, $\Delta T = T_{clear} - T_{cloud}$, represented by error bars in Figure 4 is always small, <2 °C. This hysteresis is inherent in turbidity measurements because the time needed for precipitate nucleation at $T_{cloud} < T_\tau$ and for its dissolution at $T_{clear} > T_\tau$ increases substantially when T_{clear} and T_{cloud} approach T_τ. A small hysteresis suggests that the protein condensed phase is a liquid phase rather than a crystal. Indeed, the precipitation of IgG that we observed experimentally in the presence of PEG occurred nearly instantly when the temperature was below T_{cloud}. In contrast, crystallization near the liquidus line typically exhibits a substantial time lag due to the slow nucleation process. These observations confirm that the PEG-induced precipitation is a liquid–liquid phase separation (LLPS).

In the turbidity measurements, we measure the LLPS temperature at a fixed protein concentration. In the solubility measurements, we measure protein concentration in the supernatant at a fixed temperature. These two types of measurements should produce the same results. In Figure 5, we evaluate the consistency between the coexistence curves measured by solubility and turbidity methods. In this figure, we show ascending part of the $T-C$ phase diagrams, where the temperature is plotted versus the IgG concentration at a fixed PEG concentration. As expected for LLPS, the results of solubility measurements and those of turbidity measurements

dx.doi.org/10.1021/mp400521b | *Mol. Pharmaceutics* 2014, 11, 1391−1402

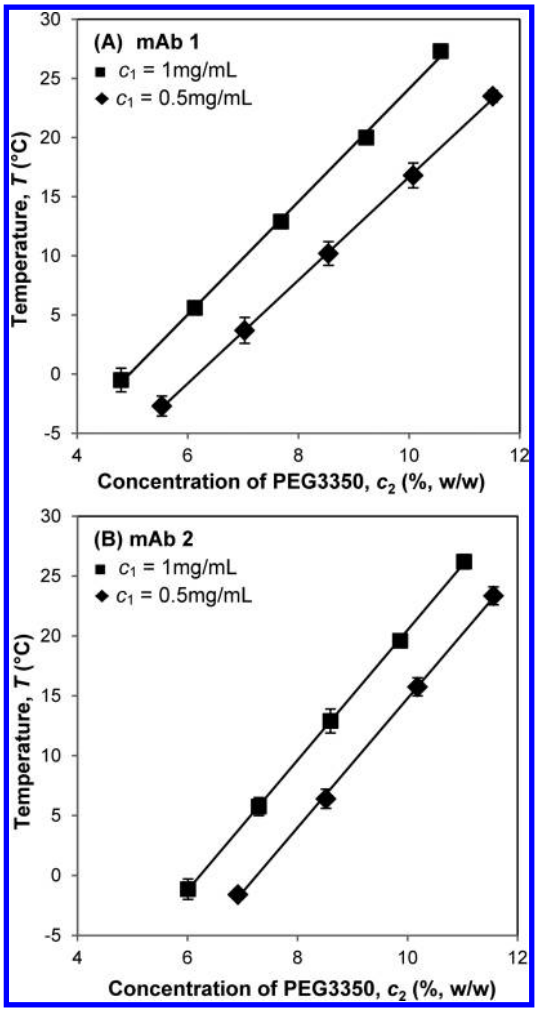


Figure 4. Condensation temperature, T_τ, as a function of PEG concentration at fixed protein concentration determined in turbidity measurement. The lower and upper ends of error bars represent respectively the clouding temperature, T_{cloud}, at which the precipitation begins and the clearing temperature, T_{clear}, at which the precipitates dissolve. The average of T_{cloud} and T_{clear} is taken as the T_τ. The solid lines are the linear regression fittings of T_τ 's.

fall onto the same $T-C$ phase boundary (typically a concave downward curve over the whole range of protein concentration) within experimental error. This consistency also indicates that LLPS is observed in the solubility measurement.

3.3. PEG-Induced LLPS under Various Solution Conditions. The depletion forces introduced by PEG produce a purely entropic interprotein attraction which universally applies to different proteins and solution conditions. (See Supporting Information section I for a brief description of the depletion force.) PEG molecules are excluded from the contact area between two proteins in close proximity to each other (Figure S1 in the Supporting Information). This absence (depletion) of PEG from the contact area results in unbalanced osmotic PEG pressure on the outsides of the proteins in contact and thus produces the effective attraction between these proteins. It is also important to note that, being excluded from the contact area between interacting proteins, PEG per se has little effect on native interprotein interactions.

PEG-induced LLPS of IgGs can be observed in solutions which have various pHs, ionic strength, cosolvents, and adjuvants. The amount of PEG required for inducing LLPS at a given temperature and protein concentration depends on the strength of "native" attractive interactions between proteins. The stronger the attraction, the lower is the PEG concentration needed. In sufficiently concentrated solutions of some IgGs, e.g., mAb 1, LLPS can be observed without PEG,[5,29] but usually the attraction between IgGs is too weak to cause LLPS above the freezing point, especially at low protein concentrations. Then, the amount of PEG needed to induce phase separation can be used as a measure of the strength of attractive interactions between IgG molecules. Therefore, PEG-induced LLPS provides a tool for evaluating the colloidal stability of an IgG under different solution conditions. Below are three examples of utilizing this approach.

First, we compared the colloidal stability of mAb2 in isotonic PBS and in the formulation buffer (20 mM acetate buffer at pH near 5) optimized for long-term storage of this pharmaceutical IgG. Figure 6 shows that the amount of PEG required for inducing IgG precipitation in the formulation buffer is much higher (~20%) than the amount of PEG required for precipitation in PBS (~10%) (see Figure 3B). Presumably, the enhanced colloidal stability of IgG in the formulation buffer is related to the fact that the protein molecules are highly positively charged at pH near 5 (the isoelectric point of mAb 2 is equal to 7.2, which is the pH of PBS) and thus strongly repel each other.

Second, we compared the colloidal stability of IgGs in CHO (Chinese hamster ovary) cell culture media used for monoclonal antibody production. LLPS in IgG solutions was induced with 8% PEG3350. As shown in Figure 7, the rank ordering of solubility of mAb 1, mAb 2, mAb 3, and mAb 4 in the centrifuge clarified cell culture fluid (pH 6.9 ± 0.1) is essentially the same as that in PBS (pH 7.2). This result suggests that PBS might be a viable surrogate for the experimental study of protein stability in cell culture media.

Lastly, we studied the PEG-induced LLPS in IgG mixture solutions. To increase throughput capacity of detecting pharmaceutical IgGs with low colloidal stability it might be advantageous to observe PEG-induced precipitation in mixtures of several IgGs at once. Here, we studied a mixture of mAb 1 and mAb 2 as an example. In pure IgG solutions, precipitation of mAb 1, and not mAb 2, can be induced with 6% PEG3350 at 0 °C (Figure 8A). With the same amount of PEG, coprecipitation of mAb 1 and mAb 2 can be induced in the mixture solution at 0 °C (Figure 8B). This result suggests that there is an attractive cross-interaction between mAb 1 and mAb 2 and the average magnitude of the mAb 1−mAb 2 interaction and the mAb 2−mAb 2 interaction is comparable to that of the mAb 1−mAb 1 interaction. This implication is directly confirmed by measuring the pairwise interaction using QLS measurements (Figure S2 in the Supporting Information). In the QLS measurements, the diffusion coefficients, D, of the IgGs were measured as a function of protein concentration. The diffusion coefficients, D_0, of the IgGs in the infinitely dilute solution were deduced by extrapolation to zero protein concentration. In Figure S2 in the Supporting Information, the normalized diffusion coefficients, $\tilde{D} = D/D_0$, of pure mAb 1 and mAb 2, as well as their 1:1 mixture, are plotted as a function of IgG concentration. At relatively low protein concentration, the slope $d\tilde{D}/dc_1$ qualitatively signifies pairwise net interprotein interaction: a negative sign of the slope indicates an attraction, a positive sign usually indicates repulsion, and the magnitude of the slope indicates the strength of interprotein interaction. Thus, Figure S2 in the

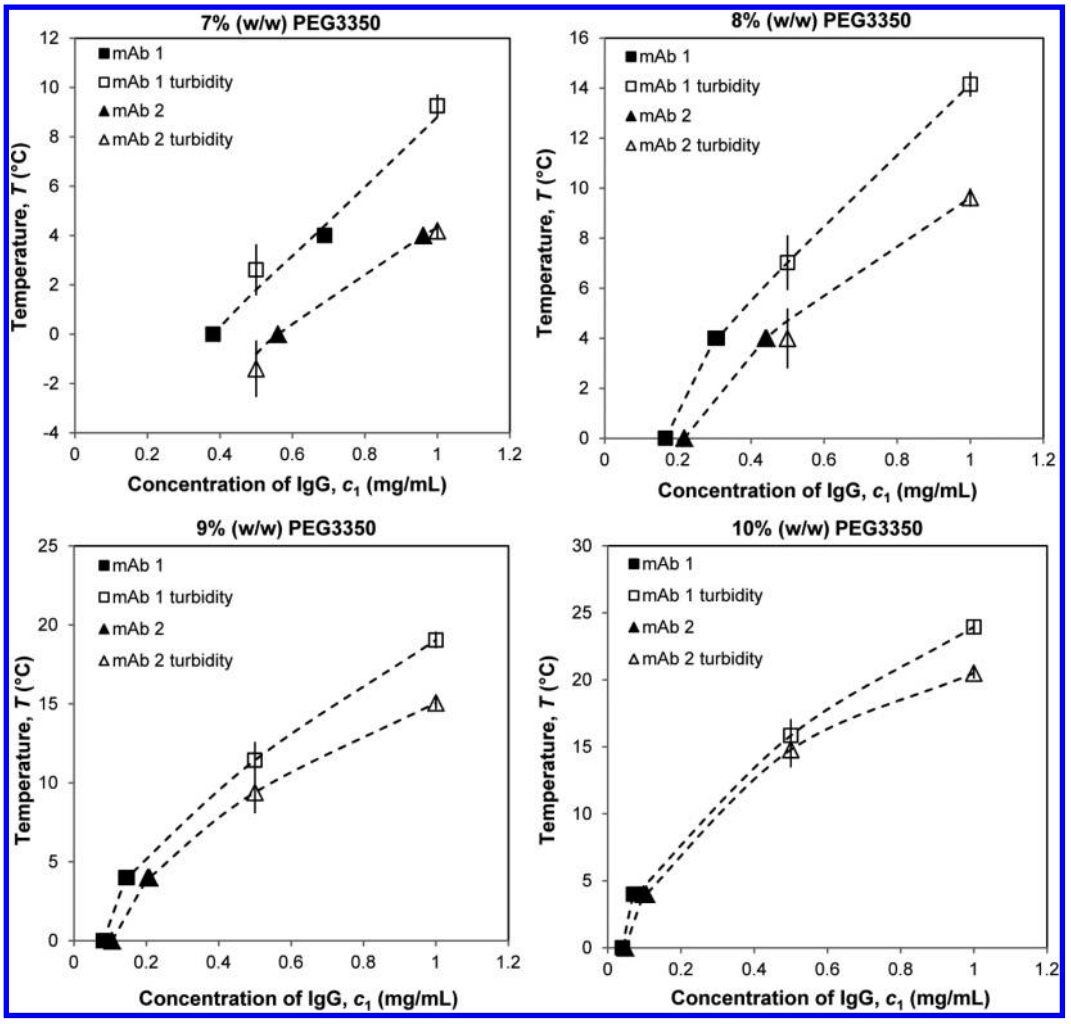


Figure 5. The $T-C$ phase boundaries (coexistence curves) of mAb 1 and mAb 2 at different PEG concentrations. The solid points are data taken from the solubility measurements shown in Figure 3. The open points are data taken from turbidity measurements shown in Figure 4. The two sets of data consistently fall on the typical concave downward $T-C$ phase boundaries shown by the dashed eye guide lines.

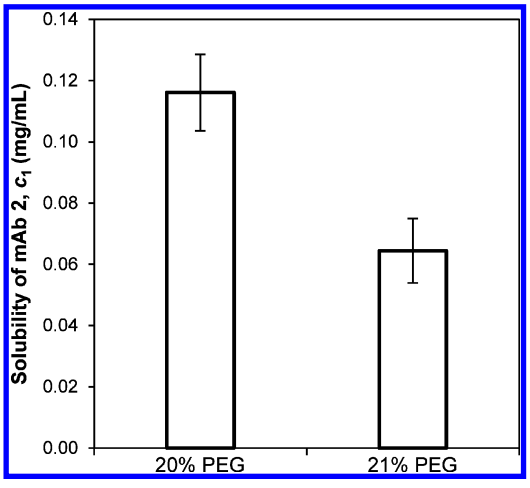


Figure 6. Solubility of mAb 2 in formulation buffer (acetate buffer at pH near 5) at two different PEG3350 concentrations incubated at 4 °C.

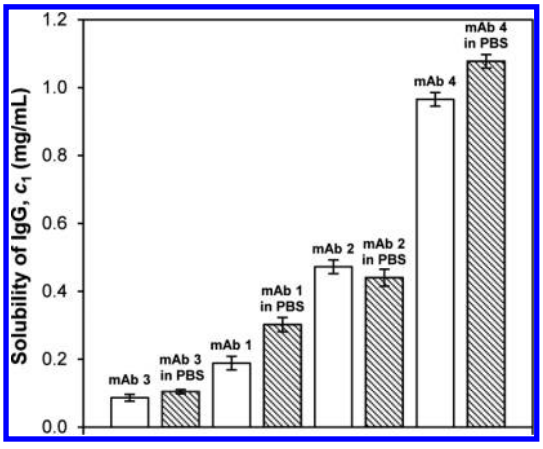


Figure 7. Comparison of the solubility of mAb 1, mAb 2, mAb 3, and mAb 4 in CHO cell culture media (open bars) and isotonic PBS (dashed bars) with 8% PEG3350 at 4 °C.

Supporting Information shows that both mAb 1 and mAb 2 have attractive interprotein interactions and that interprotein attraction between mAb 1 proteins is stronger than that between mAb 2 proteins. In the mixture, the averaged interprotein interaction is comparable to that of the pure mAb 1 solution. These results for pairwise interaction measured by QLS are consistent with conclusions derived from the PEG-induced LLPS experiments.

dx.doi.org/10.1021/mp400521b | *Mol. Pharmaceutics* 2014, 11, 1391−1402

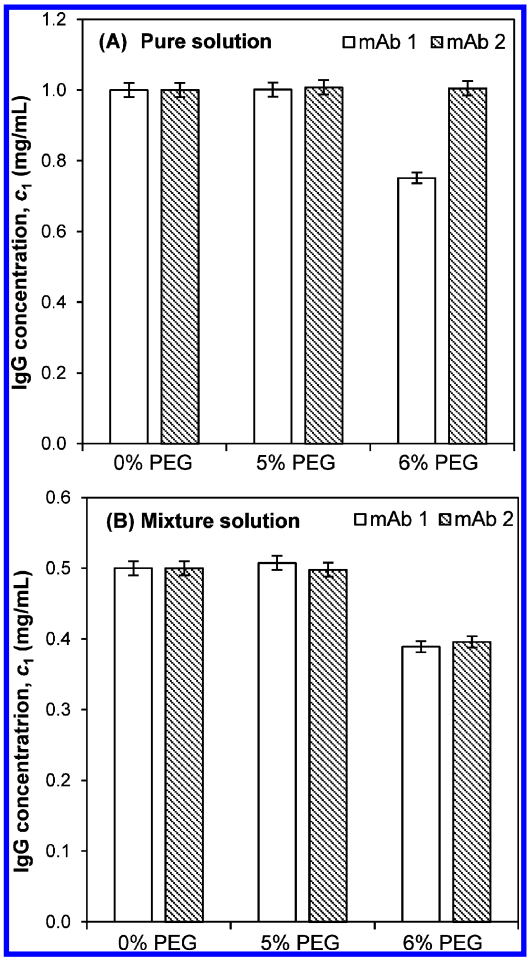


Figure 8. Protein concentrations in the supernatant after an incubation of (A) 1 mg/mL pure mAb 1 and mAb 2 solutions and (B) their 1:1 mixture solution containing 1 mg/mL total protein at 0 °C.

3.4. Effect of PEG Molecular Weight on the Precipitation of IgG. We have conducted solubility measurements to evaluate the effect of PEG molecular weight on the PEG-induced precipitation of IgGs. In these experiments, we have used PEG with different molecular weights (1500, 3350, 4600, 6000, and 8000 g/mol) to induce protein precipitation in mAb 2 solutions in PBS at 4 °C. The protein concentration in the supernatants was measured at different initial PEG concentrations (Figure 9). It is shown in Figure 9 that, at the same temperature and PEG concentration, the solubility of IgG decreases as the molecular weight of PEG increases. This result is in accordance with the dependence of depletion force on PEG molecular weight. PEG with larger molecular weight produces larger depletion interactions between protein molecules.[13,36]

4. DISCUSSION

4.1. PEG-Induced LLPS As a Tool for Evaluation of Protein Colloidal Stability. LLPS in protein solutions is caused by the net attractive interprotein interactions which are sufficiently weak to permit reorientation of protein molecules in the condensed phase. The attractive interactions can also cause crystallization and/or colloidal aggregation (i.e., aggregation not associated with protein structure changes) of proteins in sufficiently concentrated solutions. In protein solutions, when the range of interprotein interactions is short relative to the size

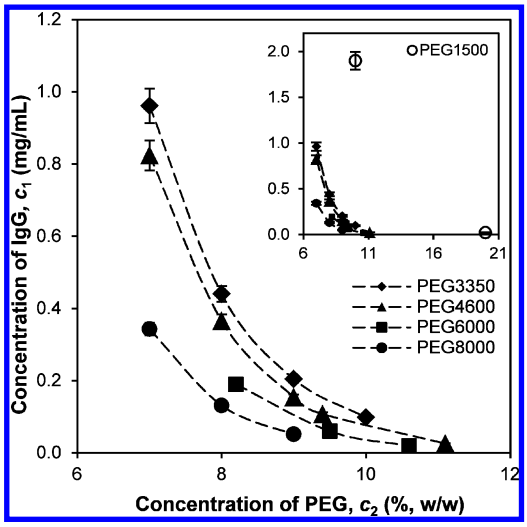


Figure 9. Measurements of mAb 2 precipitation induced by PEG with different molecular weight in PBS at 4 °C. The protein concentration in the supernatant is plotted versus the PEG concentration in the samples. The data points for PEG1500 are shown in the inset for the readability of other data. The axes of the inset are in the same units as the main figure.

of the protein molecules, LLPS is thermodynamically metastable with respect to crystallization and aggregation.[7,27−29] In other words, under solution conditions resulting in LLPS, crystallization and/or aggregation of proteins can also take place. However, crystallization and aggregation often are limited by slow nucleation kinetics and do not occur within a short period of time. That allows for an experimental observation of LLPS. It follows that IgGs with a high propensity to undergo LLPS may possibly crystallize or aggregate even without the presence of PEG. Clearly, the possibility that crystallization and/or aggregation of a particular IgG may occur under given solution conditions is of great importance not only for long-term storage but also because various sample handling processes may facilitate nucleation of crystals or aggregates. The colloidal stability of an IgG solution depends on both the inherent properties of the IgG and the solution conditions (including pH, ionic strength, salts, cosolvents, and other excipients). We discuss here the use of PEG-induced LLPS measurements for high-throughput evaluation of the colloidal stability of different IgGs under different solution conditions.

LLPS in a protein−PEG solution is fully characterized by the coexistence surface in the three-dimensional space of state variables: IgG concentration c_1, PEG concentration c_2, and solution temperature T (assuming that all other solution components partition evenly in the two coexisting phases). For both the concentrated and the dilute coexisting phases of a particular IgG, when two of these state variables are fixed, the third one is also fixed. Therefore, we can determine the location of the coexistence surface by measuring any one of these state variables in the equilibrated coexisting phase, while keeping the other two quantities fixed.

With this principle in mind, three types of PEG-induced LLPS experiments can be used to test the colloidal stability of IgGs. These experiments include the following: (1) the turbidity measurements at constant protein concentration c_1 and PEG concentration c_2; (2) the "PEG titration", i.e., measurements of the minimal concentration of PEG needed to induce precipitation at given T and c_1; and (3) the solubility

measurements at constant T and c_2, i.e., the main method used in this article. From the perspective of fundamental physics, these experiments are equivalent. However, the three methods are quite different technically. In the turbidity measurements, one of the state variables, temperature, is changed until phase separation takes place resulting in clouding of the sample. The advantage of this method is that it is easy to locate the LLPS phase boundary with high precision as the whole range of temperature (from ∼−8 to 40 °C) can be screened within a relatively short period of time (∼30 min). Furthermore, the sample does not need to reach equilibrium after precipitation commences and the sample temperature can be easily changed back and forth allowing for accurate determination of T_τ, as described in the Experimental Section. Similarly, in the PEG titration method, the PEG concentration can be increased at a fixed temperature until transition occurs. However, to change PEG concentration at a constant c_1 is much less convenient than changing the temperature.

The solubility method seems to be more preferable in industrial applications, since it can be used for high-throughput measurements of samples with very low protein concentrations and it does not require an optical setup needed for the turbidity measurements. In solubility measurements, the change in concentration of the protein in solution occurs by itself in the process of phase separation after the homogeneous sample has been brought into a thermodynamically unstable state by the addition of PEG and reduction of temperature. Though this method requires time for the two coexisting phases to reach thermodynamic equilibrium, it is more conducive to high-throughput measurements. Indeed, multiple samples can be incubated and centrifuged simultaneously, leaving the concentration measurement as the only required operation per individual sample. Thus, the solubility method is ideal for evaluating the colloidal stability of IgG solutions in industrial applications when the number of therapeutic candidates is large, and a high-throughput capability is desired. Furthermore, the solubility method can easily be applied to solutions containing multiple IgGs. Measurement of PEG-induced precipitation of IgG mixtures not only assesses the propensity of individual IgGs to undergo colloidal aggregation but also characterizes the cross-interaction between different proteins. The solubility of an IgG in a mixture is not well-defined since the equilibrium concentration of an IgG in the supernatant depends on initial concentrations of all precipitating IgGs due to their cross-interaction. Therefore, PEG-induced precipitation in mixtures provides a qualitative test of the colloidal stability of IgGs, rather than a quantitative test as in the case of pure IgG solutions. However, for industrial applications, PEG-induced precipitation in IgG mixtures may prove to be very useful because it allows screening many proteins simultaneously. The precipitating IgGs among many candidates in the mixture must include the potentially unstable (less soluble) IgGs which then can be quickly identified. A similar idea was previously discussed for the ammonium sulfate precipitation method.[9]

When using the solubility method, the possibility of crystallization or aggregation should be taken into consideration. It is known that LLPS is thermodynamically metastable with respect to crystallization, which is usually kinetically unfavorable and slow. In the solubility measurement, protein solutions need to be incubated at constant temperature for relatively long time in order to reach equilibrium. If crystals form during the incubation time, the measured solubility would be lower than that determined in the turbidity measurement under the same solution conditions.[7,29] In our study, none of the IgGs crystallized readily. Obviously, an IgG that easily crystallizes has low colloidal stability. However, it is not physically meaningful to compare the solubility in crystallization to that defined in LLPS. Therefore, when an IgG shows exceptionally low solubility in the solubility measurements, it is important to verify the absence of crystals by using either light microscope or comparison with the turbidity measurements. At the same time, identification of "crystallizable" IgGs could be valuable both for research and for the development of novel, crystalline formulations.

As we can see from Figure 3 and Figure 5, the absolute difference between the solubility of different IgGs is most prominent at lower PEG concentrations. This observation is a trivial reflection of the fact that, at high PEG concentration, all IgGs have low solubility and their solubility differences are therefore also small. This result suggests that, in the practical application, the minimal concentration of PEG sufficient to cause precipitation should be used in the PEG-induced LLPS test. Since all IgG molecules have a similar geometry, the magnitude of the depletion force is approximately the same for all IgGs. Furthermore, the major portions of IgGs have the same amino acid sequence, and therefore the relative variation in the energy of overall interprotein interaction is expected to be small. Thus, at a protein concentration of 1 mg/mL, the PEG3350 concentrations between 7% and 10% used in this work are expected to be an optimal range for the PEG-induced precipitation test of the colloidal stability of most antibody solutions at pH near 7.

4.2. A Phenomenological Theory for PEG-Induced Solubility Shift in Dilute IgG Solutions. In this work, we have focused our attention on the observation of LLPS at low IgG concentrations. Such experiments are of particular interest for evaluating interprotein interactions and colloidal stability of IgG solutions in practical applications. Furthermore, LLPS at very low protein concentrations (far away from the critical point) produces a very dilute protein-poor phase in equilibrium with a very dense condensed phase. In this limiting case, the dilute phase is ideal, PEG concentration in the condensed phase is negligible, and the condensed phase itself is incompressible. Consequently, the solubility as a function of PEG concentration is given by an equation with two phenomenological parameters, ε_B and $\Delta\nu$ (for derivation see Supporting Information section II):

$$\ln\left(\frac{\nu_0 N_A}{M_1} c_1^I\right) = -\frac{\varepsilon_B}{kT} - \Delta\nu\frac{\Pi_2}{kT} \tag{1}$$

Here, c_I^1 is the protein mass concentration in the supernatant (i.e., the solubility), M_1 is the molecular weight of the protein, N_A is Avogadro's number, k is the Boltzmann constant, T is the absolute temperature, and ν_0 is the volume per protein molecule in the condensed phase. With the incompressible approximation, ν_0 is a constant for similarly shaped IgGs and its value is determined to be ∼227 nm^3 (see Supporting Information for detailed discussion). Π_2 is the osmotic pressure of PEG, which can be calculated knowing PEG concentration and molecular weight using an semiempirical equation of state:[41]

$$\frac{\Pi_2}{kT} = \frac{N_A\rho}{M_2} c_2\left[1 + 0.49\left(\frac{c_2}{c_2^*}\right)^{5/4}\right] \tag{2}$$

Table 1. Volumes of Depletion Layer $\Delta\nu$ Excluded for PEG3350 by an IgG Molecule and the Binding Energy of IgGs in the Precipitates at 0 and 4 °C

	mAb 3	mAb 1	mAb 2	mAb 5	mAb 4
$\Delta\nu$ (nm^3)					
0 °C	93 ± 9	103 ± 6	112 ± 8	115	116 ± 4
4 °C	111 ± 9	105 ± 6	105 ± 5		123 ± 9
$N_A\varepsilon_B$ (kJ/mol)					
0 °C	16 ± 1	12.2 ± 0.5	10.9 ± 0.7	9.4	9.0 ± 0.4
4 °C	13.2 ± 0.7	10.9 ± 0.5	10.1 ± 0.4		7.1 ± 0.7

Table 2. Volumes of Depletion Layer $\Delta\nu$ for PEG with Different Molecular Weights and the Binding Energy of mAb 2 in the Precipitates at 4 °C

PEG	PEG1500	PEG 3350	PEG4600	PEG6000	PEG8000
$\Delta\nu$ (nm^3)	30 ± 6	110 ± 20	110 ± 20	130 ± 20	160 ± 30
$N_A\varepsilon_B$ (kJ/mol)	10 ± 1	10 ± 1	11.9 ± 0.9	11.6 ± 0.9	11.7 ± 0.9

where c_2 is the weight fraction of PEG in the solution, $\rho \cong 1$ g/mL is the density of the solution, and M_2 is the molecular weight of PEG. Here, $c_2^* \equiv \rho\sigma^{-0.8}/\tilde{\nu}_2$ is the scaling factor for the dilute−semidilute crossover concentration of PEG, where σ is the number of monomer units in a PEG molecule and $\tilde{\nu}_2 = 0.825$ mL/g is the partial specific volume of PEG.[41]

With eq 1 in mind, we plotted the experimental data shown in Figure 3 as the logarithm of the solubility, $\ln(c_1^I/c_0)$, versus the reduced osmotic pressure of PEG, Π_2/N_AkT (Figure S3 in the Supporting Information). c_0 is 1 mg/mL to make the argument of logarithm being dimensionless. In Figure S3 in the Supporting Information, $\ln(c_1^I/c_0)$ is proportional to Π_2/N_AkT, and the lines of the data points for different IgGs are essentially parallel but located at different places. Thus, these two parameters in eq 1 can be determined from the PEG-induced LLPS experiments for given PEG and IgG solution. $\Delta\nu$ is the magnitude of the slope of the lines in Figure S3 (Supporting Information) divided by N_A, and it has the dimension of volume. ε_B is the negative of the intercept at $\Pi_2 = 0$ less the constant, $\ln(\nu_0 N_A c_0/M_1)$.

As explained in the Supporting Information, $\Delta\nu$ is a difference between solvent volumes made inaccessible to PEG by a protein molecule in the dilute and in the condensed phase. Since $\Delta\nu$ is the proportionality coefficient for the dependence of protein solubility on osmotic pressure of PEG, it characterizes the effect of PEG on interprotein interaction. The larger $\Delta\nu$ is, the stronger is the PEG's effect on inducing LLPS. In Figure S3 in the Supporting Information, the lines of the data points for different IgGs have very similar slopes. The corresponding values of $\Delta\nu$ for PEG3350 are listed in Table 1. $\Delta\nu$ values for the different IgGs at different temperatures are all the same within the experimental error (~108 nm^3 with a standard deviation of ~8%). Observation of a universal value of $\Delta\nu$ is consistent with two facts: first, all IgGs have a common molecular geometry; second, the depletion interaction is the major effect of PEG on the IgGs in our experiments, and specific interactions between PEG and the antibodies are negligible.

The depletion interaction caused by PEG should depend on the size (molecular weight) of PEG.[36] Indeed, we expect that in the dilute phase the volume of the depletion layer scales linearly with the PEG size (Figure S1 in the Supporting Information) Using the experimental data shown in Figure 9 (see Figure S4 in the Supporting Information), the values of $\Delta\nu$ for PEG with different molecular weights are determined (Table 2). In Figure S5 in the Supporting Information, $\Delta\nu$ for PEGs with different molecular weights is plotted versus the gyration radius, R_g, of PEG. R_g of PEG is calculated using the relation $R_g \cong 0.0287M_2^{0.55}$.[13] The dependence of $\Delta\nu$ on R_g shown in Figure S5 in the Supporting Information can be described by $\Delta\nu = AR_g - \delta_0$, where the slope and the intercept of the linear fitting are determined to be $A = 55 \pm 7$ nm^2 and $\delta_0 = 50 \pm 20$ nm^3. (See Supporting Information section III for the physical interpretation of parameters of A and δ_0.) In the framework of depletion interaction, this empirical equation can be applied to calculate $\Delta\nu$ from PEG molecular weight for the PEG−IgG solutions. With $\Delta\nu$ known, the dependence of IgG solubility on the molecular weight and concentration of PEG at a given temperature can be predicted using eq 1.

We now consider the parameter ε_B in eq 1. This parameter reflects the intrinsic colloidal stability of the IgG solution without the presence of PEG. When LLPS occurs in dilute solutions, the protein concentration in the condensed phase is very high and the protein molecules are closely packed.[5] If we consider the concentrated phase as nearly incompressible (as is the case in the solid crystalline phase), a binding energy, ε_B, of IgGs (analogous to that in crystals) can be defined as the excess chemical potential of IgG in the condensed phase due to the interprotein interactions. Using the solubility data (Figure S3 in the Supporting Information), we have calculated values of ε_B for different IgGs (Table 1). The binding energy ε_B characterizes the propensity of a particular IgG to undergo LLPS. In Table 1, the values of ε_B of different IgGs follow the inversed order of their solubility at a fixed temperature. In Table 2, we also reported ε_B for an IgG determined in experiments with different PEGs. The values of ε_B are essentially the same when taking into account the uncertainty produced by the osmotic pressure equation. This consistency implies the validity of the approximation of an incompressible protein-dense phase in our experiments. For a given PEG, volumes ν_0 and $\Delta\nu$ are universal for all identically shaped IgGs. Therefore, using the values for ν_0 and $\Delta\nu$ reported here and eq 1 the binding energy ε_B for any IgG at a particular solution condition can be computed from a single PEG precipitation experiment.

When using eq 1 to calculate ε_B, it is important to keep in mind two considerations. First, eq 1 is derived in the limit of dilute solutions where the approximation of incompressible liquid condensed phase is valid. At high protein concentrations, ν_0 in eq 1 varies with the initial protein concentration and can

only be modeled with the full knowledge of packing configurations of protein molecules in the condensed phase. One way to evaluate the applicability of eq 1 is to perform the PEG-induced LLPS experiments at two different initial protein concentrations and check the consistency between the values of ε_B computed from the two experiments. Another consideration in applying eq 1 is that the universality of $\Delta\nu$ for a PEG with given molecular weight was deduced from the depletion force caused by PEG. In principle, proteins can have specific interaction with PEG.[42] In this case, eq 1 still can be used to calculate ε_B, but the value of $\Delta\nu$ needs to be determined for the specific protein by conducting the PEG-precipitation experiments at different PEG concentrations. Since IgGs within the same subclass have the same size, shape, and amino acids in most parts of the molecule, universality of $\Delta\nu$ is expected to hold for most members within the subclass. The IgGs in our experiments belong to the human IgG2 subclass. The IgG molecules from other subclasses also have similar size and shape to IgG2, thus $\Delta\nu$ is expected to have a similar value when the depletion force is the major effect of PEG. Also, within the framework of depletion force, $\Delta\nu$ should not be sensitive to the solution conditions (e.g., pH and ionic strength). In future studies, it would be valuable to evaluate the universality of $\Delta\nu$ among different IgG subclasses and under different solution conditions. Establishing the range of universality of $\Delta\nu$ and the suitable protein concentration range can help to reduce the number of experiments needed for a reliable PEG-induced LLPS assay.

4.3. Binding Energy and the Colloidal Stability of Protein Solutions. The binding energy calculated from eq 1 is the effective potential energy of a protein molecule in the liquid condensed phase. This binding energy is a measure of the overall attractive interprotein interaction. A large positive value of ε_B indicates a strong attractive interaction. The attractive interaction also leads to crystallization and colloidal aggregation. As we discussed above, the liquid protein condensates formed in LLPS are metastable and have higher chemical potential than solid protein condensates. In other words, the binding energy ε_B for a condensed liquid phase provides a lower estimate on the binding energy in crystals and colloidal aggregates. With larger binding energies crystallization or colloidal aggregation can occur at a much broader range of conditions, including at higher temperature than that required for LLPS. While in the short term solubility measurements, crystallization, and colloidal aggregation may not happen due to the slow nucleation kinetics, it is much more likely to take place during the long-term storage of the IgG solutions. Nucleation can also be facilitated during the manufacturing and formulation processes due to solution exposure to various interfaces, such as container inner surfaces or air. Furthermore, the accumulation of chemically modified proteins during the long-term storage could cause an increase of the attractive interprotein interactions. From this perspective, the proteins having larger initial attraction would be more susceptible to aggregation over time. Thus, ε_B is an important characteristic that can be used to compare the colloidal stability of different IgG solutions. Since the interprotein interaction depends on both the protein surface chemistry and the solution conditions, the binding energy can also be used to evaluate the colloidal stability of proteins under various formulation conditions such as pH, ionic strength, and excipients. Under a stable formulation condition, the binding energy can have negative values which suggest an overall repulsive interprotein interaction. When the repulsive interaction is too strong, e.g., at pH far away from p*I* and low ionic strength, it is conceivable that the PEG-induced depletion force would not be enough to cause LLPS even at very high PEG concentration (e.g., >30% w/w). However, in this case, colloidal stability should not be of concern and the formulation efforts may be directed to prevent aggregation caused by protein unfolding.

5. CONCLUSIONS

In this work, we have shown that liquid−liquid phase separation can be widely observed in IgG solutions. We demonstrated experimentally that such phase separation can be induced by PEG at IgG concentrations even below 1 mg/mL, and measured the solubility (the concentration of the dilute phase) of a number of IgGs at various PEG concentrations and temperatures. We have found that our experimental data can be well described by a simple equation assuming that the condensed phase is essentially incompressible. This equation involves two parameters: the difference between the volumes excluded for PEG by the IgG molecule in the dilute and the condensed phases, $\Delta\nu$, and the binding energy of IgG in the condensed liquid phase, ε_B. We have demonstrated that $\Delta\nu$ for a given PEG size is the same for all IgGs in our experiments, and have deduced from the experimental data the phenomenological equation for computing $\Delta\nu$ for PEGs with different molecular weights. The binding energy, ε_B, is a measure of the net attractive interactions between IgG molecules. It is a lower estimate of the binding energy in crystals and colloidal aggregates and can serve as a comparative characterization tool of the colloidal stability of IgG under given solution conditions.

Knowing $\Delta\nu$, ε_B can be determined by a single solubility measurement. Large positive value of ε_B signifies strong attractions between IgG molecules and consequently a low colloidal stability. Thus, the solubility test serves as a quantitative assay of the colloidal stability of the protein solutions. Our experimental work and theoretical analysis provides a foundation for using the PEG-induced LLPS test to assess the likelihood of protein precipitation during various stages of formulation and as a result of the potential protein degradation on long-term storage. This solubility test of the colloidal stability is conducted directly under the solution conditions used in production or storage of the protein. It is also feasible to use the solubility measurements in the mixtures of multiple IgG pharmaceutical candidates as a high-throughput screening test in the early stage of drug development.

■ ASSOCIATED CONTENT

Supporting Information

Brief description of the depletion force induced by PEG, derivation of eq 1, discussion of dependence of $\Delta\nu$ on PEG molecular weight, and figures depicting mechanism of the interprotein depletion force induced by PEG, normalized diffusion coefficients of pure mAb 1 and mAb 2, $\ln(c_1/c_0)$ of different IgGs as a function of PEG osmotic pressure, and the difference between the volumes of depletion layers in the dilute phase and the condensed phase, $\Delta\nu$, as a function of the radius of gyration of PEG. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Department of Physics, 77 Massachusetts Avenue, Cambridge, MA 02139. Tel: 617-253-4828. Fax: 617-253-0359. E-mail: benedek@mit.edu.

Author Contributions

‡Y.W and R.F.L. contributed equally to this work.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We thank Jifeng Zhang, Bruce D. Mason, and Thomas M. McNerney (all of Amgen Inc.) and Olutayo Ogun (MIT) for reagent supply and technical support and thank Douglass D. Banks, Gerd R. Kleemann, Michael J. Treuheit, and Ping Yeh (all of Amgen Inc.) for helpful discussions.

REFERENCES

(1) Dübel, S. *Handbook of Therapeutic Antibodies: Technologies, Emerging Developments and Approved Therapeutics*; John Wiley & Sons: New York, 2010.

(2) Chen, S.; Lau, H.; Brodsky, Y.; Kleemann, G. R.; Latypov, R. F. The use of native cation-exchange chromatography to study aggregation and phase separation of monoclonal antibodies. *Protein Sci.* **2010**, *19*, 1191−1204.

(3) Nishi, H.; Miyajima, M.; Nakagami, H.; Noda, M.; Uchiyama, S.; Fukui, K. Phase separation of an IgG1 antibody solution under a low ionic strength condition. *Pharm. Res.* **2010**, *27*, 1348−1360.

(4) Mason, B. D.; Zhang-van Enk, J.; Zhang, L.; Remmele, R. L., Jr.; Zhang, J. Liquid-liquid phase separation of a monoclonal antibody and nonmonotonic influence of Hofmeister anions. *Biophys. J.* **2010**, *99*, 3792−3800.

(5) Wang, Y.; Lomakin, A.; Latypov, R. F.; Benedek, G. B. Phase separation in solutions of monoclonal antibodies and the effect of human serum albumin. *Proc. Natl. Acad. Sci. U.S.A.* **2011**, *108*, 16606−16611.

(6) Trilisky, E.; Gillespie, R.; Osslund, T. D.; Vunnum, S. Crystallization and liquid-liquid phase separation of monoclonal antibodies and fc-fusion proteins: Screening results. *Biotechnol. Prog.* **2011**, *27* (4), 1054−1067.

(7) Mason, B. D.; Zhang, L.; Remmele, R. L., Jr.; Zhang, J. Opalescence of an IgG2 monoclonal antibody solution as it relates to liquid-liquid phase separation. *J. Pharm. Sci.* **2011**, *100*, 4587−4596.

(8) Lewus, R. A.; Darcy, P. A.; Lenhoff, A. M.; Sandler, S. I. Interactions and phase behavior of a monoclonal antibody. *Biotechnol. Prog.* **2011**, *27*, 280−289.

(9) Banks, D. D.; Latypov, R. F.; Ketchem, R. R.; Woodard, J.; Scavezze, J. L.; Siska, C. C.; Razinkov, V. I. Native-state solubility and transfer free energy as predictive tools for selecting excipients to include in protein formulation development studies. *J. Pharm. Sci.* **2012**, *101*, 2720−2732.

(10) Yamniuk, A. P.; Ditto, N.; Patel, M.; Dai, J.; Sejwal, P.; Stetsko, P.; Doyle, M. L. Application of a kosmotrope-based solubility assay to multiple protein therapeutic classes indicates broad use as a high-throughput screen for protein therapeutic aggregation propensity. *J. Pharm. Sci.* **2013**, *102* (8), 2424−2439.

(11) Gibson, T. J.; McCarty, K.; McFadyen, I. J.; Cash, E.; Dalmonte, P.; Hinds, K. D.; Dinerman, A. A.; Alvarez, J. C.; Volkin, D. B. Application of a high-throughput screening procedure with PEG-induced precipitation to compare relative protein solubility during formulation development with IgG1 monoclonal antibodies. *J. Pharm. Sci.* **2011**, *100*, 1009−1021.

(12) Curtis, R. A.; Ulrich, J.; Montaser, A.; Prausnitz, J. M.; Blanch, H. W. Protein-protein interactions in concentrated electrolyte solutions. *Biotechnol. Bioeng.* **2002**, *79*, 367−380.

(13) Bhat, R.; Timasheff, S. N. Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols. *Protein Sci.* **1992**, *1*, 1133−1143.

(14) Fesinmeyer, R. M.; Hogan, S.; Saluja, A.; Brych, S. R.; Kras, E.; Narhi, L. O.; Brems, D. N.; Gokarn, Y. R. Effect of ions on agitation- and temperature-induced aggregation reactions of antibodies. *Pharm. Res.* **2009**, *26*, 903−913.

(15) Bhambhani, A.; Kissmann, J. M.; Joshi, S. B.; Volkin, D. B.; Kashi, R. S.; Middaugh, C. R. Formulation design and high-throughput excipient selection based on structural integrity and conformational stability of dilute and highly concentrated IgG1 monoclonal antibody solutions. *J. Pharm. Sci.* **2012**, *101*, 1120−1135.

(16) Thomson, J. A.; Schurtenberger, P.; Thurston, G. M.; Benedek, G. B. Binary liquid phase separation and critical phenomena in a protein/water solution. *Proc. Natl. Acad. Sci. U.S.A.* **1987**, *84*, 7079−7083.

(17) Taratuta, V. G.; Holschbach, A.; Thurston, G. M.; Blankschtein, D.; Benedek, G. B. Liquid-liquid phase separation of aqueous lysozyme solutions: effects of pH and salt identity. *J. Phys. Chem.* **1990**, *94*, 2140−2144.

(18) Galkin, O.; Chen, K.; Nagel, R. L.; Hirsch, R. E.; Vekilov, P. G. Liquid-liquid separation in solutions of normal and sickle cell hemoglobin. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 8479−8483.

(19) Dumetz, A. C.; Chockla, A. M.; Kaler, E. W.; Lenhoff, A. M. Protein phase behavior in aqueous solutions: crystallization, liquid-liquid phase separation, gels, and aggregates. *Biophys. J.* **2008**, *94*, 570−583.

(20) Zhang, F.; Roosen-Runge, F.; Sauter, A.; Roth, R.; Skoda, M. W. A.; Jacobs, R. M. J.; Sztucki, M.; Schreiber, F. The role of cluster formation and metastable liquid-liquid phase separation in protein crystallization. *Faraday Discuss.* **2012**, *159*, 313−325.

(21) Dorsaz, N.; Thurston, G. M.; Stradner, A.; Schurtenberger, P.; Foffi, G. Phase separation in binary eye lens protein mixtures. *Soft Matter* **2011**, *7*, 1763−1776.

(22) Annunziata, O.; Ogun, O.; Benedek, G. B. Observation of liquid-liquid phase separation for eye lens gammaS-Crystallin. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 970−974.

(23) Lomakin, A.; Asherie, N.; Benedek, G. B. Monte Carlo study of phase separation in aqueous protein solutions. *J. Chem. Phys.* **1996**, *104*, 1646−1656.

(24) Wang, Y.; Annunziata, O. Liquid-liquid phase transition of protein aqueous solutions isothermally induced by protein cross-linking. *Langmuir* **2008**, *24*, 2799−2807.

(25) Pusey, P. N.; van Megen, W. Phase behaviour of concentrated suspensions of nearly hard colloidal spheres. *Nature* **1986**, *320*, 340−342.

(26) Rascón, C.; Navascués, G.; Mederos, L. Phase transitions in systems with extremely short-ranged attractions: A density-functional theory. *Phys. Rev. B* **1995**, *51*, 14899−14906.

(27) Muschol, M.; Rosenberger, F. Liquid−liquid phase separation in supersaturated lysozyme solutions and associated precipitate formation/crystallization. *J. Chem. Phys.* **1997**, *107*, 1953−1962.

(28) Lomakin, A.; Asherie, N.; Benedek, G. B. Liquid-solid transition in nuclei of protein crystals. *Proc. Natl. Acad. Sci. U.S.A.* **2003**, *100*, 10254−10257.

(29) Wang, Y.; Lomakin, A.; Latypov, R. F.; Laubach, J. P.; Hideshima, T.; Richardson, P. G.; Munshi, N. C.; Anderson, K. C.; Benedek, G. B. Phase transitions in human IgG solutions. *J. Chem. Phys.* **2013**, *139*, 121904.

(30) Grigsby, J. J.; Blanch, H. W.; Prausnitz, J. M. Cloud-point temperatures for lysozyme in electrolyte solutions: effect of salt type, salt concentration and pH. *Biophys. Chem.* **2001**, *91*, 231−243.

(31) Wang, Y.; Lomakin, A.; McManus, J. J.; Ogun, O.; Benedek, G. B. Phase behavior of mixtures of human lens proteins Gamma D and Beta B1. *Proc. Natl. Acad. Sci. U.S.A.* **2010**, *107*, 13282−13287.

(32) Jenkins, N. Modifications of therapeutic proteins: challenges and prospects. *Cytotechnology* **2007**, *53*, 121−125.

(33) Luo, Q.; Joubert, M. K.; Stevenson, R.; Ketchem, R. R.; Narhi, L. O.; Wypych, J. Chemical modifications in therapeutic protein

aggregates generated under different stress conditions. *J. Biol. Chem.* **2011**, *286*, 25134−25144.
(34) Pande, J.; Lomakin, A.; Fine, B.; Ogun, O.; Sokolinski, I.; Benedek, G. Oxidation of gamma II-Crystallin solutions yields dimers with a high phase separation temperature. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 1067−1071.
(35) Annunziata, O.; Pande, A.; Pande, J.; Ogun, O.; Lubsen, N. H.; Benedek, G. B. Oligomerization and phase transitions in aqueous solutions of native and truncated human beta B1-Crystallin. *Biochemistry* **2005**, *44*, 1316−1328.
(36) Annunziata, O.; Asherie, N.; Lomakin, A.; Pande, J.; Ogun, O.; Benedek, G. B. Effect of polyethylene glycol on the liquid-liquid phase transition in aqueous protein solutions. *Proc. Natl. Acad. Sci. U.S.A.* **2002**, *99*, 14165−14170.
(37) Wang, Y.; Annunziata, O. Comparison between protein-polyethylene glycol (PEG) interactions and the effect of PEG on protein-protein interactions using the liquid-liquid phase transition. *J. Phys. Chem. B* **2007**, *111*, 1222−1230.
(38) Vivarès, D.; Belloni, L.; Tardieu, A.; Bonneté, F. Catching the PEG-induced attractive interaction between proteins. *Eur. Phys. J. E* **2002**, *9*, 15−25.
(39) Arakawa, T.; Timasheff, S. N. Mechanism of poly(ethylene glycol) interaction with proteins. *Biochemistry* **1985**, *24*, 6756−6762.
(40) Lau, H.; Pace, D.; Yan, B.; McGrath, T.; Smallwood, S.; Patel, K.; Park, J.; Park, S. S.; Latypov, R. F. Investigation of degradation processes in IgG1 monoclonal antibodies by limited proteolysis coupled with weak cation-exchange HPLC. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2010**, *878*, 868−876.
(41) Cohen, J. A.; Podgornik, R.; Hansen, P. L.; Parsegian, V. A. A phenomenological one-parameter equation of state for osmotic pressures of PEG and other neutral flexible polymers in good solvents. *J. Phys. Chem. B* **2009**, *113*, 3709−3714.
(42) Bloustine, J.; Virmani, T.; Thurston, G. M.; Fraden, S. Light scattering and phase behavior of lysozyme-poly(ethylene glycol) mixtures. *Phys. Rev. Lett.* **2006**, *96*, 087803.

Quantitative Evaluation of Colloidal Stability of Antibody Solutions using PEG-induced Liquid-Liquid Phase Separation

I. Depletion force induced by PEG

Liquid-liquid phase separation of IgG's results from the net attractive inter-protein interaction. The stronger the attraction, the higher is the LLPS temperature. For most IgG's, the attractive interaction is so weak that LLPS cannot be practically observed as it theoretically occurs at temperature below the freezing point of the solution (~–7°C observed in our experiments). PEG introduces additional isotropic attractive inter-protein interactions, due to the so called depletion force (*1-5*), and thereby induces LLPS of IgG's at elevated temperatures.

The depletion force results from the steric exclusion of the PEG molecules from the vicinity of the protein molecules. This exclusion is characterized by the so called depletion layer around a protein molecule which is not accessible to the center of mass of PEG molecules (Fig. S1). Clearly, the thickness of the depletion layer is proportional to the linear size of the PEG molecule. For most proteins, the specific attractive interactions between PEG and protein are negligible as compared to the depletion force. The Helmholtz free energy of a protein-PEG mixture solution of volume V is a sum of the protein free energy (F_1), and the PEG free energy (F_2):

$$F = F_1\left(N_{1,}, V, T\right) + F_2(N_2, V_{PEG}, T) \qquad \text{Eq.S1}$$

where N_1 and N_2 are, respectively, the numbers of protein and PEG molecules and V_{PEG} is the volume accessible to PEG molecules. Let us assume that two protein molecules come into proximity of each other so that their depletion layers overlap by volume δV. Then the volume accessible to PEG is increased to $V_{PEG} + \delta V$ and the free energy of the system is changed by $\frac{\partial F_2}{\partial V_{PEG}} \cdot \delta V = -\Pi_2 \cdot \delta V$, where Π_2 is the osmotic pressure of PEG molecules. The depletion force can be interpreted as resulting from the osmotic pressure exerted on the protein molecules from the outside which is not compensated by the osmotic pressure from within the

overlap volume δV. Clearly, for non-spherical molecules such as IgG's the depletion force is a function of the mutual orientation of the IgG molecules. Furthermore, PEG is a deformable polymer which cannot be completely excluded even from a small gap between IgG molecules. Therefore, the accurate description of the overlap volume δV between IgG molecules is quite complicated. Nevertheless, as in the case of spherical proteins, the effective volume of the depletion layer per IgG molecule can be evaluated as the product of surface area of the depletion layer times an effective thickness proportional to the radius of the PEG molecule.

II. LLPS in dilute protein-PEG mixture solutions

LLPS in very dilute solutions is amenable to rather simple qualitative theoretical analysis. The simplification is brought about by the fact that far below the critical temperature a very dilute phase coexists with a very concentrated phase.(*6*) The dilute phase is then essentially an ideal solution, while the concentrated phase can be approximately considered incompressible, i.e. its properties, including density (or volume per protein molecule) and the chemical potential of the protein molecules, are nearly independent of the osmotic pressure. This is analogous to water-vapor equilibrium, where at room temperature, far below the critical temperature of 374 °C, vapor (dilute phase) is an ideal gas while water (dense phase) is practically incompressible.

In a pure protein solution, the free energy of the protein in two coexisting phases *I* and *II* is $F_1(N_1, V, T) = F_1^I(N_1^I, V^I, T) + F_1^{II}(N_1^{II}, V^{II}, T)$. Here N^I and V^I is the number of protein molecules and volume of phase *I* (dilute), where $N_1^{II} = N_1 - N_1^I$ and $V^{II} = V - V^I$ is the number of protein molecules and volume of phase *II* (condensed). At equilibrium, the free energy should be at a minimum with respect to the variables, N_1^I and V^I. That leads to equality of the chemical potentials $\mu^I = \frac{\partial F^I}{\partial N_1^I} = \frac{\partial F^{II}}{\partial N_1^{II}} = \mu^{II}$ and of the osmotic pressures $-\frac{\partial F^I}{\partial V^I} = -\frac{\partial F^{II}}{\partial V^{II}}$ in both coexisting phases. When the dilute phase is ideal and the condensed phase is incompressible, we can write the chemical potential of the dilute and condensed phases as: $\mu^I = \mu^o + kTln(v_0 C_1^I)$, and $\mu^{II} = \mu^o - \varepsilon_B$ where v_0 and ε_B are, respectively, the volume per protein molecule and the binding energy in the condensed phase. μ^o is the standard chemical

potential of the IgG molecule in the incompressible condense phase without the presence of inter-protein interactions. Then the molar concentration of the dilute phase $C_1^I \equiv \frac{N_1^I}{V^I}$ is given by the Arrhenius law, $C_1^I = \frac{1}{v_0} exp\left(-\frac{\varepsilon_B}{kT}\right)$. The equality of osmotic pressures in both phases is established by minimal adjustments of the density of the nearly incompressible condensed phase.

As we discussed before, LLPS does not normally occur in pure IgG solutions at low concentrations above the solvent freezing temperature. Thus, PEG always has to be added so that LLPS can occur in dilute IgG solutions. Again, far below the critical point, when the dilute phase is ideal and the condensed phase is practically incompressible, PEG partitions exclusively into the dilute phase. In our experiments reported here, the initial protein concentration in the sample is 1 mg/mL. The coexisting liquid precipitates should have a protein concentration of at least 300 mg/mL.(*6, 7*) At such high protein concentrations, the IgG molecules are closely packed together leaving little room for PEG molecules, and the PEG concentration in the precipitates is essentially zero. Therefore, addition of PEG has little effect on properties of the condensed phase including the chemical potential of protein μ^{II}, which remains a function of the temperature only. If PEG is effectively excluded from the condensed phase, the volume accessible to PEG can be written as $V_{PEG}^I = V - V^{II} - N_1^I \nu$, where ν is the volume excluded for PEG by one individual protein molecule in the dilute phase. Since the overlap of the depletion layers in the dilute phase is negligible, ν includes the volume of protein itself and depletion layer around it. The volume for the condensed phase can be written as $V^{II} = N_1^{II} \cdot v_0$. Thus, $V_{PEG}^I = V - N_1 v_0 - N_1^I \Delta\nu$, where $\Delta\nu = \nu - v_0$ is the difference of the PEG-excluded volume per protein molecule in the dilute and the condensed phases. Clearly, $\nu > v_0$ since in the condensed phase the depletion layers around protein molecules overlap significantly. Thus, $\Delta\nu$ always has a positive value. According to Eq.S1 the contribution of the PEG to the chemical potential of the protein in the dilute phase is $\frac{\partial F_2}{\partial N_1^I} = \frac{\partial F_2}{\partial V_{PEG}} \frac{\partial V_{PEG}}{\partial N_1^I}$. Since $\frac{\partial F_2}{\partial V_{PEG}} = -\Pi_2$,

$$\mu^I = \mu^o + kTln(v_0 C_1^I) + \Pi_2 \Delta\nu \qquad \text{Eq. S2}$$

Since in our approximation the chemical potential of the condensed phase, $\mu^{II} = \mu^{o} - \varepsilon_B$ is not affected by the presence of PEG, we conclude that in equilibrium μ^{I} also remains the same and the protein solubility, in mg/mL units, in the presence of PEG is described by:

$$\ln(\nu_0 C_1^I) = -\Delta\nu \frac{\Pi_2}{kT} - \frac{\varepsilon_B}{kT} \qquad \text{Eq. S3}$$

Note that term $\Pi_2 \Delta\nu$ is in fact the work done by osmotic pressure of PEG as it expands into volume $\Delta\nu$ freed upon incorporation of the protein molecule into the condensed phase. By converting the molar concentration of protein, C_1^I, to the mass concentration, c_1^I, we obtain Eq.1 from Eq.S3.

III. Dependence of Δν on PEG molecular weight

By definition, $\Delta\nu = \nu - \nu_0$ characterizes the difference between the excluded volume for PEG by one IgG molecule in the coexisting dilute phase (supernatant) and condensed phase (liquid precipitates). In the dilute phase, the protein molecules are far from each other, and the depletion layers for different protein molecules do not overlap. Thus, the volume of the depletion layer is equal to its thickness, *r*, multiplied by its surface area, *a*. Since IgG's have a similar size and shape, the value of *a* can be assumed to be a constant for all IgG's. The thickness of the depletion layer, *r*, is proportional to the gyration radius of PEG, R_g, i.e. $r = \xi R_g$. Thus, $\nu = \Omega + AR_g$, where Ω is the volume of a protein molecule and $A \equiv a\xi$. In the condensed phase, protein molecules are closely packed. In this case, the depletion layers around different protein molecules are highly overlapped, and the total depletion zone includes essentially all empty spaces that are not occupied by the protein molecules. In other words, ν_0 is simply the volume per IgG molecule in the condensed phase. When LLPS occurs in dilute protein solutions, the protein concentration in the condensed phase is quite high and depends very little on the initial protein concentration, temperature and the PEG concentration. Therefore, in our experiments, ν_0 can be considered as a constant independent of the solution conditions. The approximation of constant A and ν_0 has been validated by the universal value of $\Delta\nu$ for

different IgG's measured in the experiments with PEG3350 described in the last section. With above considerations, the expression of $\Delta\nu$ can be rewritten as:

$$\Delta\nu = AR_g - \delta_0 \qquad \text{Eq.S4}$$

where $\delta_0 = \nu_0 - \Omega$ is the "empty space" per protein molecule, not occupied by the IgG itself, in the condensed phase. Fitting the experimental data we have determined that $A = 55\pm7$ nm^2 and $\delta_0 = 50\pm20$ nm^3 for the PEG-IgG solutions. These values of the parameters A and δ_0 are result of extrapolation and should not be interpreted as actual area of antibody surface and empty volume per antibody in condensed phase. We have also presented in Fig. S5 the values of $\Delta\nu$'s calculated using another phenomenological expression for osmotic pressure described in reference (*8*). While the $\Delta\nu$'s calculated using these two equations are essentially consistent with each other, the slope and the intercept (70 nm^2 and 90 nm^3) derived using the second osmotic pressure equation are somewhat different from the results obtained using Eq.4 in the main text. Furthermore, Eq.S4 itself is only justifiable when $R_g << R$ where R is the characteristic curvature of the protein surface. The PEG-IgG pair is of course not in this limit. Thus, Eq.S4 is actually a phenomenological equation that reflects experimentally observed linear relationship between $\Delta\nu$ and R_g. The phenomenological nature of Eq.S4 does not diminish its practical value for predicting the dependence of IgG solubility (for LLPS) on the PEG molecular weight and concentration.

Interestingly, on the qualitative level, the numerical values of the parameters A and δ_0 make sense in terms of physical properties of IgG and PEG molecules in the protein condensed phase. Indeed, the value of parameter ξ defined as the ratio of the thickness of the depletion layer to the gyration radius of PEG in the dilute region, $\xi = r/R_g$, has been determined to be ~0.5 at low PEG concentrations in the solutions of quasi-spherical proteins.(*1, 2*) At high PEG concentrations, ξ is expected to be smaller than 0.5 due to the repulsions between PEG molecules.(*9*) The area of the depletion layer, a, around the non-spherical IgG molecules is difficult to evaluate. On the other hand, an equivalent sphere with the same volume as an IgG molecule should have the minimum surface area. It is reasonable to speculate that a is larger than this minimum surface area, ~152nm^2 which is estimated using the molecular weight $M_1 \cong$

150,000Da and molecular density $\rho_1 \cong 1.4$ g/mL for IgG's (*10*). With all the approximations made in the estimation, the value of A (55 nm^2) determined in our experiment is surprisingly close to the value of 152× 0.5 = 76 nm^2. From the value of δ_0 (50nm^3), we can estimate the solution volume per IgG molecule in the protein condensed phase, $v_0 = \Omega + \delta_0 \cong 227$nm^3, where the molecular volume of IgG is $\Omega = M_1/N_A\rho_1$. This value of v_0 is very close to that of close-packing spheres (*11*) having the same density as the protein molecule, v_{CS} = 239 nm^3. Even though the shape of IgG molecules is far from spherical, the small value of v_0 indicates the dense packing of IgG molecules in the condensed phase. This is expected for the LLPS in very dilute protein solutions. In addition, if PEG is partially incorporated into the condensed phase, the volume excluded for PEG in the condensed phase, δ_0, will be diminished.

Fig.S1. A schematic mechanism of the inter-protein depletion force induced by PEG. In (A), the overlap of depletion layers (gray areas) creates additional free volume (white areas) accessible for the PEG molecules. Thus, the spatial configuration of protein molecules in (A) is entropically more favorable than that in (B).

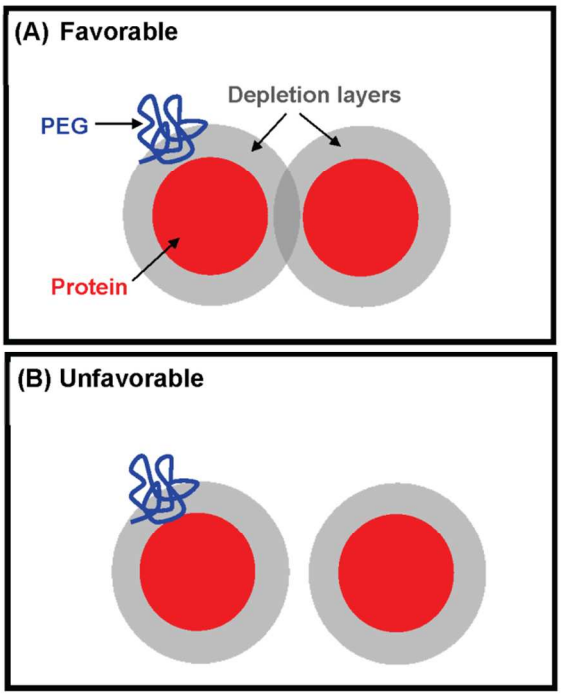


Fig. S2. Normalized diffusion coefficients of pure mAb 1 and mAb 2, as well as their 1:1 mixture, measured by QLS as a function of protein concentration.

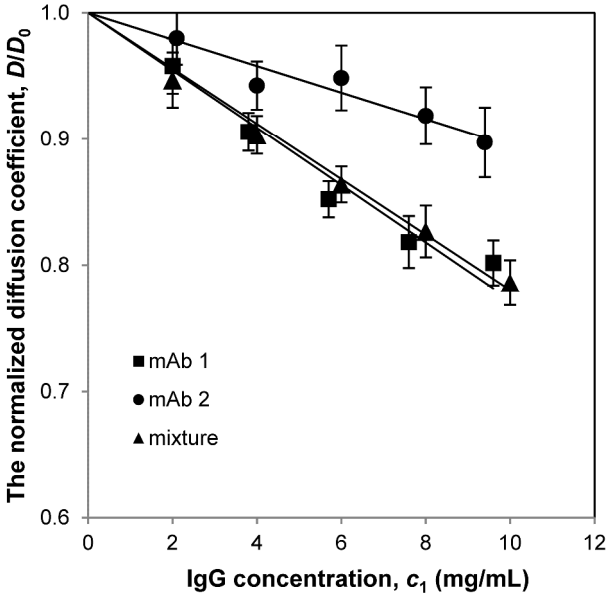


Fig. S3. $\ln(c_1/c_0)$ of different IgG's as a function of PEG osmotic pressure Π_2 at 0 °C and 4 °C using data of the solubility measurement shown in Fig. 3. All correlation coefficients, R^2, of the linear regressions are higher than 0.99.

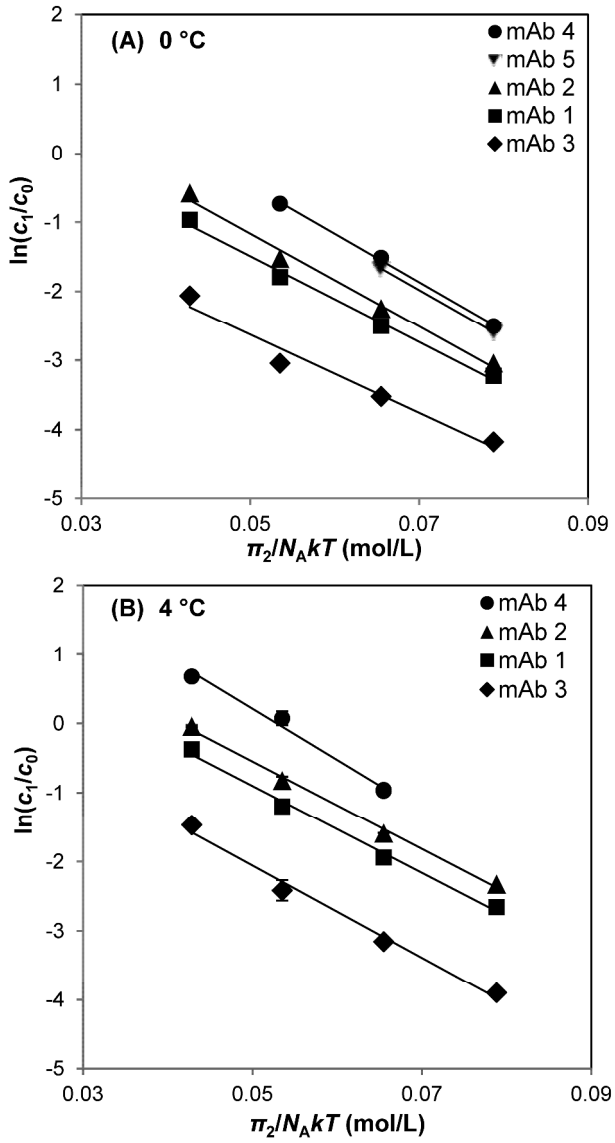


Fig.S4. $\ln(c_1/c_0)$ of IgG mAb 2 as a function of PEG osmotic pressure Π_2 for PEG with different molecular weight at 4 °C using data of the solubility measurement in Fig. 9. The data points for PEG1500 are shown in the inset for the readability of other data. The axis's of the inset are in the same units as the main figure. All correlation coefficients, R^2, of the linear regressions are higher than 0.99.

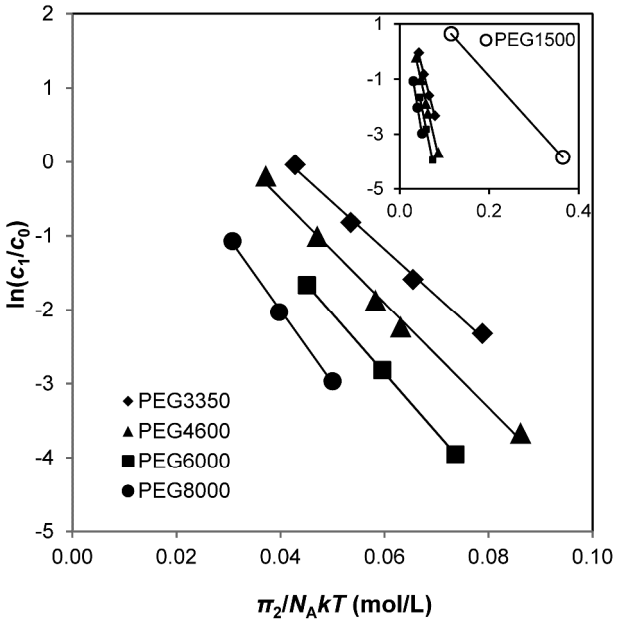
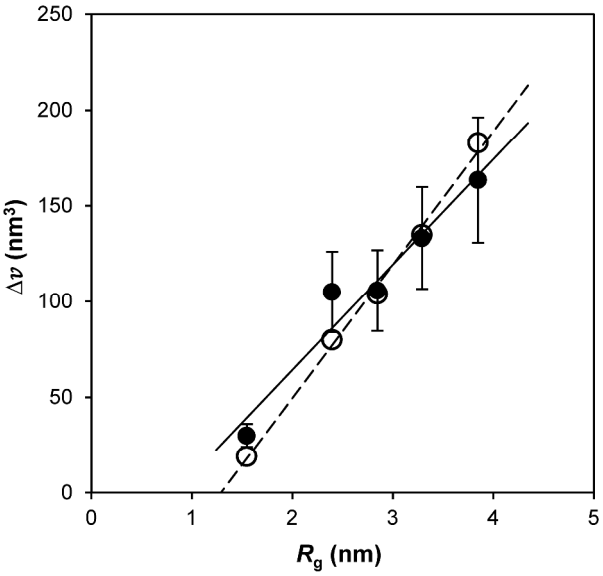


Fig.S5. The difference between the volumes of depletion layers in the dilute phase and the condensed phase, Δv, as a function of the radius of gyration of PEG. The solid circles are calculated with the equation of state used in this paper (Eq. 2). The open circles are calculated using the expression of osmotic pressure of PEG solutions described in ref (*8*). The solid and the dashed lines respectively represent the linear fitting of the solid and the open circles. R^2=0.95 and 1.00 respectively for fitting the solid and the open circles.



Reference:

1. Annunziata, O., Asherie, N., Lomakin, A., Pande, J., Ogun, O., and Benedek, G. B. (2002) Effect of polyethylene glycol on the liquid-liquid phase transition in aqueous protein solutions, *Proc Natl Acad Sci U S A 99*, 14165-14170.
2. Wang, Y., and Annunziata, O. (2007) Comparison between protein-polyethylene glycol (PEG) interactions and the effect of PEG on protein-protein interactions using the liquid-liquid phase transition, *J Phys Chem B 111*, 1222-1230.
3. Bhat, R., and Timasheff, S. N. (1992) Steric exclusion is the principal source of the preferential hydration of proteins in the presence of polyethylene glycols, *Protein Science 1*, 1133-1143.
4. Vivarès, D., Belloni, L., Tardieu, A., and Bonneté, F. (2002) Catching the PEG-induced attractive interaction between proteins, *Eur. Phys. J. E 9*, 15-25.
5. Arakawa, T., and Timasheff, S. N. (1985) Mechanism of poly(ethylene glycol) interaction with proteins, *Biochemistry 24*, 6756-6762.
6. Wang, Y., Lomakin, A., Latypov, R. F., and Benedek, G. B. (2011) Phase separation in solutions of monoclonal antibodies and the effect of human serum albumin, *Proc Natl Acad Sci U S A 108*, 16606-16611.
7. Wang, Y., Lomakin, A., Latypov, R. F., Laubach, J. P., Hideshima, T., Richardson, P. G., Munshi, N. C., Anderson, K. C., and Benedek, G. B. (2013) Phase transitions in human IgG solutions, *J Chem Phys 139*, 121904.
8. Kulkarni, A. M., Chatterjee, A. P., Schweizer, K. S., and Zukoski, C. F. (2000) Effects of polyethylene glycol on protein interactions, *The Journal of Chemical Physics 113*, 9863-9873.
9. Zhivkov, A. M. (2007) Dependence of depletion layer thickness on polymer concentration determined by Vincent's pragmatic and Donath's electrophoretic theories, *Journal of colloid and interface science 313*, 122-127.
10. Fischer, H., Polikarpov, I., and Craievich, A. F. (2004) Average protein density is a molecular-weight-dependent function, *Protein Science 13*, 2825-2828.
11. Torquato, S., Truskett, T. M., and Debenedetti, P. G. (2000) Is Random Close Packing of Spheres Well Defined?, *Physical Review Letters 84*, 2064-2067.