Light scattering and phase behavior of Lysozyme-PEG mixtures

J. Bloustine¹, T. Virmani¹, G. M. Thurston², and S. Fraden¹

¹Complex Fluids Group, Martin Fisher School of Physics, Brandeis University, Waltham, MA 02454
²Department of Physics, Rochester Institute of Technology, Rochester, NY 14623

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Measurements of liquid-liquid phase transition temperatures (cloud points) of mixtures of a protein (lysozyme) and a polymer, poly(ethylene glycol) (PEG) show that the addition of low molecular weight PEG stabilizes the mixture whereas high molecular weight PEG was destabilizing. We demonstrate that this behavior is inconsistent with an entropic depletion interaction between lysozyme and PEG and suggest that an energetic attraction between lysozyme and PEG is responsible. In order to independently characterize the lysozyme/PEG interactions, light scattering experiments on the same mixtures were performed to measure second and third virial coefficients. These measurements indicate that PEG induces repulsion between lysozyme molecules, contrary to the depletion prediction. Furthermore, it is shown that third virial terms must be included in the mixture’s free energy in order to qualitatively capture our cloud point and light scattering data. The light scattering results were consistent with the cloud point measurements and indicate that attractions exist between lysozyme and PEG.

The addition of small polymers to a dispersion of large colloids can result in precipitation or crystallization of the colloids. Even when the sole colloid/polymer interaction is steric repulsion, an attraction between a pair of colloids is generated by the exclusion of polymer molecules from the region between the colloids. This entropic effect is known as depletion attraction [1, 2]. The water soluble polymer poly(ethylene glycol) (PEG) has been utilized extensively to induce protein crystallization [3]. Can the mechanism of PEG induced crystallization be explained by the purely entropic depletion effect? This work addresses the related question of whether or not PEG induces attraction between lysozyme molecules.

In addition to a liquid to crystal transition, protein solutions exhibit a metastable liquid-liquid phase transition when cooled [1, 2]. This phase transition temperature is termed the cloud point \( T_{\text{cloud}} \) since at this temperature a transparent protein solution becomes turbid as liquid droplets of high protein concentration form in a liquid of lower protein concentration. The effect of added PEG on \( T_{\text{cloud}} \) was studied for several of the \( \gamma \)-crystallin proteins by Benedek and co-workers who found that \( T_{\text{cloud}} \) of \( \gamma S \) crystallin increases as PEG is added [3] in agreement with depletion attraction whereas for \( \gamma D \) crystallin their results [4] indicate a departure from pure depletion. Galkin and Vekilov [5] studied lysozyme/PEG mixtures and found that the effect of PEG molecular weight on \( T_{\text{cloud}} \) depended on the solution’s ionic strength so no clear evaluation of the depletion effect could be made. Because the cloud point depends on salt type and concentration [1, 2], all our measurements were performed in the same solution conditions.

We model the thermodynamics of a protein/polymer mixture by expanding the excess Gibbs free energy \( G \) of a two component solution relative to that of the solvent in powers of the densities of the two independent solutes, labelled 1 and 2.

\[
g = \frac{G}{k_B T} = \rho_1 \ln \rho_1 + \rho_2 \ln \rho_2 + B_{11} \rho_1^2 + 2B_{12} \rho_1 \rho_2 + B_{22} \rho_2^2 + C_{111} \rho_1^3 + 3C_{112} \rho_1^2 \rho_2 + \ldots \tag{1}
\]

In Eq. (1) \( \rho_i = N_i / [\text{Volume}] \); \( k_B \) is Boltzmann’s constant; \( T \) is the absolute temperature; \( B_{ij} \) [Volume] are the second virial coefficients and \( C_{ijk} \) [Volume²] are the third virial coefficients. Virial coefficients are related to integrals of the potential of mean force between molecules and depend on \( T \).

A bidisperse hard sphere mixture was used as the reference system for lysozyme/PEG mixtures because hard sphere systems provide a natural scale for virial coefficients. Hard spheres cannot interpenetrate, but have no other interactions. The virial coefficients in Eq. (1) for a bidisperse hard sphere mixture are [6]:

\[
 B_{11}^{\text{HS}} = (16\pi / 3) r_1^3, \quad B_{12}^{\text{HS}} = 2\pi / 3 (r_1 + r_2)^3, \tag{2}
\]

\[
 C_{111}^{\text{HS}} = \frac{5}{16} (B_{11}^{\text{HS}})^2, \quad C_{112}^{\text{HS}} = \frac{8\pi^2}{27} r_1^3 (r_1^3 + 6r_1^2 r_2 + 15r_1 r_2^2 + 8r_2^3) \tag{3}
\]

where \( r_i \) are the hard sphere radii.

The ratio of lysozyme to PEG measured hydrodynamic radii, \( r_H \), in our experiments varied in the range \( 3 \geq r_H^{\text{PEG}} / r_H^{\text{lysozyme}} \geq 0.8 \). Therefore we need to account for the fact that PEG molecules, which are nearly as large or larger than lysozyme molecules, are not spherical and wrap partially around proteins thereby reducing the polymer/protein excluded volume. This is done by defining an ideal effective polymer radius \( r_{\text{eff}} \) which is smaller than the polymer’s radius of gyration \( r_g \). Eisenriegler et al. [7] found a closed formula for computing \( r_{\text{eff}} \) from the protein radius and \( r_g \) (see Fig. 6 in ref. [7]). Our procedure was to equate the protein’s radius with its...
measured $r_H$ whereas the process to determine the PEG’s $r_g$ is described in the following paragraph.

The PEG radii of gyration for the molecular weights employed here were too small to measure with static light scattering because $r_g$ is much less than the wavelength of light. In order to determine $r_g$, we therefore first measured $r_H$ and then used the relation $r_g/r_H = 1.488M_2^{0.012}$ where $M_2$ [g mol$^{-1}$] is the PEG molecular weight. This should be compared to the theoretical value, $r_g/r_H = 1.56$, for a polymer in a good solvent [14].

For lysozyme we found: $r_H^\text{ly} = 2.2$ nm which falls within the range of previously reported values [15]. We measured $r_H^{\text{PEG}} = 0.75$ and 2.7 nm for PEG1k and 8k respectively where the manufacturer’s stated value for $M_2$ is designated with the nomenclature PEGnk, meaning PEG n x 10$^3$ [g mol$^{-1}$]. The procedure outlined above yields $r_H = 1.2$ and 3.8 nm for PEG1k and 8k.

We measured the cloud point temperature of lysozyme solutions as an approximation of the spinodal decomposition temperature since the cloud point closely tracks the spinodal [16]. Starting from the free energy, we calculate how the spinodal temperature changes with added polymer concentration at fixed protein concentration. In all that follows the subscript 1 refers to lysozyme and the subscript 2 refers to PEG. A two component mixture at fixed concentration undergoes spinodal decomposition when the temperature reaches $T_{sp}$ defined by [17]:

$$f(T_{sp}, \rho_1, \rho_2) = \frac{\partial^2 g}{\partial \rho_1^2} \frac{\partial^2 g}{\partial \rho_2^2} - \left( \frac{\partial^2 g}{\partial \rho_1 \partial \rho_2} \right)^2 = 0$$

Imposing the constraints that the solution remains on the spinodal curve when polymer is added and that the protein concentration is constant one finds $\partial T_{sp}/\partial \rho_2 = -(\partial f/\partial \rho_2)/(\partial f/\partial T)$. From Eq. (11), one then obtains to first order in $\rho_1$:

$$\lim_{\rho_2 \to 0} \frac{\partial T_{sp}}{\partial \rho_2} = -\frac{3C_{112} + 2(B_{12} + 3\rho_1C_{112})^2}{\partial B_{11}/\partial T + 3\rho_1^2 \frac{\partial C_{111}}{\partial \rho_1}}$$

In all that follows we have set $\partial C_{111}/\partial T = 0$ since experimentally $C_{111} = 0$ at all temperatures.

We define the numerator of Eq. (11) to be $\gamma$. The PEG molecular weight dependence enters Eq. (11) only through the virial coefficients. From Eqs. (2) (4) (5) and (6), it can be shown that for a hard sphere mixture $\gamma > 0$ for all sphere sizes $r_1$ and $r_2$ and concentrations $\rho_1$. In the limit of small polymers ($q = r_2/r_1 \ll 1$) we find that $2\gamma/(B_{11}^\text{HS}/4)^2 = 12q^3$ in agreement with scaled particle depletion models [18].

In order to explore the dependence of $T_{\text{cloud}}$ on PEG molecular weight further, virial coefficients were determined from light scattering experiments on the same lysozyme/PEG mixtures and then compared with predictions of the depletion theory. Kirkwood & Goldberg [20] showed that the excess light scattering of a two solute system ($R_{1+2}$) over that of a single solute system ($R_2$) can be written as:

$$\frac{K_{c_1}}{R_{1+2} - R_2} = \alpha + \beta \times c_1$$

Here $K = 2(\pi n_o n_i)^2/N_A \lambda^4$ where $n_o$ is the solvent refractive index, $n_i = \frac{n}{\lambda}$ is the refractive index increment of solute $i$, $N_A$ is Avogadro’s number, $\lambda$ is the wavelength of the incident radiation in vacuum, $c_i$[g mL$^{-1}$] is the solute weight concentration and $R$ is the Rayleigh ratio.

$\alpha$ and $\beta$ depend on the added polymer properties ($M_2$, $n_2$), concentration $c_2$, and the protein/polymer interaction $21$:

$$\alpha = \frac{1}{M_1} + c_2 \frac{4M_2n_2B_{12}}{M_1n_1}$$

$B_{12}$ is obtained from measurements of $\alpha$ as a function of polymer concentration $c_2$ shown in Fig. 2.

The coefficient $\beta$ is given by

$$\beta = 2B_{11} + 2c_2 \left[ (3C_{112} - 2B_{12}^2M_2) + \frac{2(3C_{112} + 2B_{112} + 2B_{12})M_2n_2}{M_1n_1} \right]$$

$C_{112}$ is obtained from measurements of $\beta$ as a function of $c_2$, as shown in Fig. 2 since all the other quantities in Eq. (7) are determined independently.

It is possible to view the two component polymer/protein solution as an effective one component protein solution. The effective protein/protein second virial coefficient, $B_{11}^\text{eff}$, can be obtained from Eq. (7) by imagining the addition of invisible polymers ($n_2 = 0$) to the protein solution [21] [22] which yields: $B_{11}^\text{eff} = B_{11} + c_2 \left[ (3C_{112} - 2B_{12}^2M_2) \right]$. Experimentally we do not index match the polymer ($n_2 \neq 0$). Instead we measure the virial coefficients and calculate $B_{11}^\text{eff}$.

Hen egg white lysozyme was purchased from Sekagaku America. $M_1$ based on sequence is 14,400 g mol$^{-1}$. Poly(ethylene glycol) (PEG) was purchased from Sigma and Fluka.

The protein and PEG were dissolved in a 0.2M sodium phosphate buffer with NaCl 0.5M at pH 6.2 where $M \equiv \text{mol L}^{-1}$. All solutions were centrifuged at $\sim 12000 \times g$ for 1 hour and then passed through 0.2$\mu$m filters directly into precleaned scattering cuvettes. The lysozyme concentrations were measured by UV absorption using an extinction coefficient $\varepsilon_{280\text{nm}} = 2.64$ mL mg$^{-1}$ cm$^{-1}$.

Refractive index increments of lysozyme and PEG were measured using a Brookhaven Instruments differential refractometer at $\lambda = 620$ nm. For lysozyme $dn/dc = 1.85 \times 10^{-4}$ mL mg$^{-1}$ and for PEG $dn/dc = 1.34 \times 10^{-4}$ mL mg$^{-1}$ independent of $M_2$ and $T$.

Cloud point temperatures were determined by optical microscopy. Rectangular glass capillaries (0.1 mm path length, VitroCom) were filled with solution, flame sealed and then placed in a custom built temperature controlled microscope stage. The temperature at which
homogeneous nucleation of dense droplets occurred was called $T_{\text{cloud}}$. The temperature of each solution was cycled up and down at approximately $1^\circ\text{C}$ min$^{-1}$ through $T_{\text{cloud}}$ several times for each measurement. Little or no difference ($\leq 0.5^\circ\text{C}$) was observed when comparing $T_{\text{cloud}}$ obtained by cooling and heating.

The static and dynamic light scattering experiments (SLS & DLS) were performed using an ALV goniometer and correlator system in the cu polarization mode. Absolute Rayleigh ratios of aqueous solutions were determined by using pure toluene as a standard whose Rayleigh ratio is known. The lysozyme and PEG hydrodynamic radii $r_1$ were obtained from DLS measurements on dilute solutions.

Our $T_{\text{cloud}}$ measurements of lysozyme/PEG mixtures at constant ionic strength reveal a systematic trend: whereas $T_{\text{cloud}}$ increases upon the addition of high molecular weight PEG, it decreases for low molecular weight PEG as shown in Figure 1. We extracted $\lim_{p_2 \to 0} \partial T_{\text{cloud}}/\partial p_2$ from this data. Measurements of $T_{\text{cloud}}$ made at higher lysozyme concentrations near lysozyme’s critical point showed that $\lim_{p_2 \to 0} \partial T/\partial p_2$ is independent of lysozyme concentration (data not shown). The hard sphere mixture model predicts that the cloud point temperature dependence on PEG concentration cannot change sign with PEG molecular weight, i.e. $\gamma > 0$. However, our experiments reveal qualitatively different behavior. For dilute PEG concentrations, low molecular weight PEGs depress the cloud point, i.e. stabilize the solution, which is opposite to the depletion prediction, while higher molecular weight PEGs raise the cloud point, i.e. destabilize the solution.

Table I shows the PEG molecular weights obtained from SLS data. PEG1k and 8k second virial coefficients as functions of temperature were found to be equal to 0. However, our experiments reveal qualitatively different behavior. For dilute PEG concentrations, low molecular weight PEGs depress the cloud point, i.e. stabilize the solution, which is opposite to the depletion prediction, while higher molecular weight PEGs raise the cloud point, i.e. destabilize the solution.

Table I shows the PEG molecular weights obtained from DLS measurements on dilute solutions. 

$B_{11}^\text{P1k}(T)[\text{mL mol g}^{-2}] = 0.01 - (8.8 \times 10^{-5} \times T[\circ\text{C}])$ and $B_{11}^\text{P8k}(T)[\text{mL mol g}^{-2}] = 5.6 \times 10^{-3} - (9.5 \times 10^{-3} \times T[\circ\text{C}])$, which agree well with previous results. Table II shows that measured PEG second virial coefficients are the same order as those for equivalent hard spheres, implying that PEG interactions are repulsive. For lysozyme: $B_{11}(T = 20^\circ\text{C}) = -3.95 \pm 0.3 \times 10^{-4}$ mL mol g$^{-1}$, $\partial B_{11}/\partial T = 1 \times 10^{-5}$ mL mol g$^{-2}$ °C$^{-1}$ and $M_1 = 13800 \pm 500$ g mol$^{-1}$.

Equation (6) demonstrates that important information about lysozyme/PEG interactions is contained in $\alpha(c_2)$, namely $B_{12}$. The measured values of $B_{12}$ shown in Table I are consistent with previous measurements. If interference between the scattering from lysozyme and PEG was negligible then $\partial \alpha/\partial c_2 = 0$, as seen from Equation (6) with $(M_2 n_2)/(M_1 n_1) = 0$. However, Figure 2 panel a) shows that $\partial \alpha/\partial c_2 > 0$ consistent with Prausnitz and co-workers. This demonstrates that interference from lysozyme and PEG scattering may not be ignored as done by Kulkarni et al. and that $\alpha$ may not be treated as a constant. Figure 2 panel b) displays the variation of $\beta$ with PEG concentration. The solid lines through the data points are the linear fits used to obtain the values of $C_{112}$ by Equation (7) shown in Table I. We found $B_{12}$ and $C_{112}$ to be temperature independent.

Much interest has been generated by the conjecture that the second virial coefficient may be sufficient to predict protein solution phase behavior. The measured dependencies of $T_{\text{cloud}}$ and $\beta$ on PEG concentration show that third virial coefficients must be included in the free energy of lysozyme/PEG mixtures, i.e. $C_{112} \neq 0$. If $C_{112} = 0$ then Equation (7) predicts $\beta(c_2)$ shown as the
dashed lines in panel b) of Fig 2, which disagree with our data. Additionally, if $C_{112} = 0$ then Eq. (1) predicts that $\partial T_{sp}/\partial c_2$ cannot change sign as a function of PEG molecular weight, which is inconsistent with Fig 1.

Table I compares our measured values of the mixed virial coefficients, $B_{12}$ and $C_{112}$, to $B_{12}^{HS}$ and $C_{112}^{HS}$, calculated from Eqs. (2,3). The lysozyme equivalent hard sphere radius was taken to be $r_1$ and the PEG equivalent hard sphere radii were taken to be $r_{eff}$. The lysozyme/PEG mixed virial coefficients are less than those of equivalent hard sphere mixtures. Therefore, attractive interactions must exist between the protein and polymer or the repulsion must be less than that between hard spheres.

The measured virial coefficients allow us to calculate the variation of the effective lysozyme second virial coefficient with PEG concentration, $\partial B_{11}^{eff}/\partial c_2$. Depletion theory predicts that PEG induces attraction between lysozyme molecules in which case $\partial B_{11}^{eff}/\partial c_2 < 0$ as seen from Eqs. (2,3). Conversely, if $\partial B_{11}^{eff}/\partial c_2 > 0$ then PEG induces repulsion between lysozyme molecules. For PEG 8k we find $\partial B_{11}^{eff}/\partial c_2 = 0.33 \pm 0.2 \times 10^{-3}$ mL$^2$ mol$^{-2}$ and for PEG 1k we find that $\partial B_{11}^{eff}/\partial c_2 = 1.6 \pm 0.2 \times 10^{-3}$ mL$^2$ mol$^{-2}$. We conclude that adding PEG weakens the attraction between lysozyme molecules in contradiction to depletion theory.

We find experimentally $\lim_{c_2 \to 0} \partial T_{cloud}/\partial c_2 = 0.15$, $0.06 \pm 0.02 \degree C$ mg$^{-1}$ mL$^{-1}$ for PEG 1k and 8k respectively. Using Eq. (4) with the measured virial coefficients obtained independently from SLS yields $\lim_{c_2 \to 0} \partial T_{sp}/\partial c_2 = -0.03 \pm 0.05$, $0.005 \pm 0.008 \degree C$ mg$^{-1}$ mL$^{-1}$ for PEG 1k and 8k respectively. These results show that the measured virial coefficients can correctly reproduce the sign of $\lim_{c_2 \to 0} \partial T_{cloud}/\partial c_2$. As discussed previously, $\gamma > 0$ for a hard sphere mixture whereas Fig 1. shows that experimentally the sign of $\gamma$ varies with PEG molecular weight. Therefore a depletion model cannot account for the observed variation of the cloud point on polymer concentration, but the measured virial coefficients in conjunction with Eq. (1) do so qualitatively.

This paper demonstrates that the depletion theory does not describe PEG/lysozyme mixtures. Firstly, the observation that adding low molecular weight PEG depresses $T_{cloud}$ whereas high molecular weight PEG raises $T_{cloud}$ cannot be accounted for by a pure depletion model. Secondly, depletion theory predicts that adding PEG induces an attraction between lysozyme molecules whereas light scattering revealed the opposite: PEG induces repulsion between lysozyme molecules. Lysozyme/PEG interactions were characterized by virial coefficients obtained from light scattering experiments. It is demonstrated that to even qualitatively explain our data, the free energy must include third virial terms. The measured mixed virial coefficients are smaller than those predicted for an equivalent hard sphere mixture and are consistent with attractions between lysozyme and PEG.

Models of hydrogen bonding of water molecules to the PEG backbone qualitatively explain PEG’s phase behavior alone in water. Therefore we speculate that PEG can similarly form hydrogen bonds with residues on the surface of lysozyme molecules thereby creating the attraction between PEG and lysozyme. The measured virial coefficients, combined with thermodynamic theory predict the observed behavior of the cloud point demonstrating the consistency of these two independent sets of experiments. Therefore, an accurate model of the phase behavior and light scattering of lysozyme/PEG mixtures must account for both the entropic depletion effect and an energetic attraction between protein and polymer.

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* Present Address: Center for Basic Neuroscience, The University of Texas Southwestern Medical Center, Dallas, TX 75390
TABLE I: PEG molecular weights (M_2) and second virial coefficients (B_{22}) taken from light scattering data at T= 30°C, the degree of PEG polymerization (N = M_2/44) and mixed virial coefficients (B_{12}, C_{112}) for lysozyme/PEG solutions obtained from fits to α and β, Eqs. (6,7), are shown. The equivalent hard sphere values were obtained by using r_g of PEG to determine B_{22}^{HS} and r_{eff} of PEG along with r_H of lysozyme to determine B_{12}^{HS} and C_{112}^{HS} by Eqs. (2,3). The error bars came from the linear least squares fits.

|      | M_2 [10^3 g/mol] | N  | B_{22} [10^{-4} mL mol/g^2] | B_{22}/B_{22}^{HS} [10^{-4} mL mol/g^2] | B_{12} [10^{-4} mL mol/g^2] | B_{12}/B_{12}^{HS} [10^{-4} mL^2 mol/g^3] | C_{112} | C_{112}/C_{112}^{HS} |
|------|------------------|----|----------------------------|----------------------------------------|-----------------------------|------------------------------------------|---------|-----------------------|
| PEG1k| 0.97 ± 0.1       | 22 | 9.2± 1.0                   | 0.5                                    | 12.9±1.2                    | 0.34                                      | 16.1±1.3 | 0.23                 |
| PEG8k| 10.4±0.5         | 236| 3.35±0.12                  | 0.4                                    | 1.7±0.3                     | 0.089                                     | 3.13±0.2 | 0.051                |

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