Light Scattering and Phase Behavior of Lysozyme-Poly(Ethylene Glycol) Mixtures

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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 lysozyme-PEG depletion interaction and suggest that an energetic lysozyme-PEG attraction 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 data.

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 Letter 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 [4–6]. This phase transition temperature is termed the *cloud point* (T_{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_{cloud} was studied for several of the γ-crystallin proteins by Benedek and co-workers who found that T_{cloud} of γS crystallin increases as PEG is added [6] in agreement with depletion attraction, whereas for γD crystallin, their results [7] indicate a departure from pure depletion. Galkin and Vekilov [8] studied lysozyme-PEG mixtures and found that the effect of PEG molecular weight on T_{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 [9], all our measurements were performed in constant 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, labeled 1 and 2:

$$g = \frac{G}{Vk_BT} = \rho_1 \ln\rho_1 + \rho_2 \ln\rho_2 + B_{11}\rho_1^2 + 2B_{12}\rho_1\rho_2$$
$$+ B_{22}\rho_2^2 + C_{111}\rho_1^3 + 3C_{112}\rho_1^2\rho_2 + \dots, \quad (1)$$

where $\rho_i = N_i/V$ [volume^{-1}], k_B is Boltzmann's constant, T is the absolute temperature, B_{ij} [volume] are the second virial coefficients, and C_{ijk} [volume^2] are the third virial coefficients. Virial coefficients are related to integrals of the potential of mean force between molecules and are temperature dependent.

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 [10]

$$B_{11}^{HS} = (16\pi/3)r_1^3, \qquad B_{12}^{HS} = (2\pi/3)(r_1 + r_2)^3, \quad (2)$$

$$C_{112}^{HS} = (8\pi^2/27)r_1^3(r_1^3 + 6r_1^2r_2 + 15r_1r_2^2 + 8r_2^3), \quad (3)$$

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 $3 \geq r_H^{lys}/r_H^{PEG} \geq 0.8$. 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_{eff}), which is smaller than the polymer's radius of gyration r_g. Eisenriegler *et al.* [11] found a closed formula for computing r_{eff} from the protein radius and r_g (see Fig. 6 in Ref. [11]). Our procedure was to equate the protein's radius with its measured r_H. In order to determine the PEG's r_g we first measured its r_H and then used the relation $r_g/r_H = 1.48M_2^{0.012}$ [12] 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 [13]. For lysozyme we found $r_H^{lys} = 2.2$ nm, which falls within the range of previously reported values [14]. We measured $r_H^{PEG} = 0.75$ and 2.7 nm for PEG 1k and 8k, respectively, where the manufacturer's stated value for M_2 is designated as PEGnk, meaning PEG $n \times 10^3$ [g mol^{-1}]. The procedure outlined above yields $r_{eff} = 1.2$ and 3.8 nm for PEG 1k and 8k.

We measured the cloud point temperature of lysozyme solutions as a function of PEG concentration (Fig. 1) as an approximation of the spinodal decomposition temperature since the cloud point closely tracks the spinodal [5]. 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 [15]: $f(T_{sp}, \rho_1, \rho_2) = (\partial^2 g/\partial \rho_1^2) \times (\partial^2 g/\partial \rho_2^2) - (\partial^2 g/\partial \rho_1 \partial \rho_2)^2 = 0$. Imposing the constraints that the solution remains on the spinodal curve when polymer is added and that the protein concentration is constant, one finds $\partial T_{sp}/\partial \rho_2 = -(\partial f/\partial \rho_2)/(\partial f/\partial T)$. Using the virial expansion for the free energy, one then obtains to first order in ρ_1

$$\lim_{\rho_2 \to 0} \frac{\partial T_{sp}}{\partial \rho_2} = \frac{-3C_{112} + 2(B_{12} + 3\rho_1 C_{112})^2}{\partial B_{11}/\partial T + 3\rho_1 \frac{\partial C_{111}}{\partial T}}. \quad (4)$$

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. (4) to be γ. The PEG molecular weight dependence enters Eq. (4) only through the virial coefficients. From Eqs. (2)–(4), it can be shown that for a hard sphere mixture $\gamma > 0$ for all sphere sizes r_1 and r_2 and concentrations ρ_1. In the limit of small polymers ($q = r_2/r_1 \ll 1$) we find that $2\gamma/(B_{11}^{HS}/4)^2 = 12q^3$ in agreement with scaled particle depletion models [7].

In order to explore the dependence of T_{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 and Goldberg [16] 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 $Kc_1/(R_{1+2} - R_2) = \alpha + \beta \times c_1$. Here $K = 2(\pi n_0 n_1)^2/N_A \lambda^4$ where n_0 is the solvent refractive index, $n_i = \partial n/\partial c_i$ is the refractive index increment of solute i, N_A is Avogadro's number, λ is the wavelength of the incident radiation in vacuum, c[g mL^{-1}] is the solute weight concentration, and R is the Rayleigh ratio.

α and β depend on the added polymer properties (M_2, n_2), concentration c_2, and the protein-polymer interaction [16]:

$$\alpha = 1/M_1 + 4c_2 m B_{12}, \quad (5)$$

where $m = M_2 n_2/M_1 n_1$. B_{12} is obtained from measurements of α as a function of polymer concentration c_2 shown in Fig. 2. The coefficient β is given by

$$\beta = 2[B_{11}^{eff} + m c_2 (3C_{112} + 2B_{11}B_{12}M_1)], \quad (6)$$

with $B_{11}^{eff} = B_{11} + c_2[(3C_{112} - 2B_{12}^2 M_2)]$. C_{112} is obtained from measurements of β as a function of c_2, as shown in Fig. 2, since all the other quantities in Eq. (6) 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}^{eff}, can be obtained from Eq. (6) by imagining the addition of invisible polymers ($n_2 = m = 0$) to the protein solution [17,18]. Experimentally we do not index match the polymer ($n_2 \neq 0$). Instead we measure the virial coefficients and calculate B_{11}^{eff}.

Hen egg white lysozyme was purchased from Seikagaku America. M_1 based on sequence is 14 400 g mol^{-1}. PEG was purchased from Sigma and Fluka. The protein and PEG were dissolved in a 0.2 M sodium phosphate buffer with NaCl 0.5M at pH 6.2 where $M \equiv$ [mol L^{-1}]. All solutions were centrifuged at $\sim$12 000 $\times g$ for 1 h and then passed through 0.2 μm filters directly into precleaned scattering cuvettes. The lysozyme concentrations were measured by UV absorption using an extinction coefficient $\epsilon_{280 \, nm} = 2.64$ 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 $n_1 = 1.85 \times 10^{-4}$ mL mg^{-1} and for PEG $n_2 = 1.34 \times 10^{-4}$ 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_{cloud}. The temperature of each solution was cycled up and down at approximately 1 °C min^{-1} through T_{cloud} several times for each measurement. Little or no difference ($\leq$0.5 °C) was observed when comparing T_{cloud} obtained by cooling and heating. The static (SLS) and dynamic light scattering experiments (DLS) were performed using an ALV goniometer and correlator system in the vu polarization mode. Absolute Rayleigh ratios of aqueous solutions were determined by using pure toluene as a standard whose Rayleigh ratio is known [19]. The lysozyme and PEG hydrodynamic radii r_H were obtained from DLS measurements on dilute solutions [20].

Our T_{cloud} measurements of lysozyme-PEG mixtures at constant ionic strength reveal a systematic trend: whereas T_{cloud} increases upon the addition of high molecular weight PEG, it *decreases* for low molecular weight PEG as shown in Fig. 1. We extracted $\lim_{\rho_2 \to 0} \partial T_{cloud}/\partial \rho_2$ from this data. Measurements of T_{cloud} made at higher lysozyme concen-
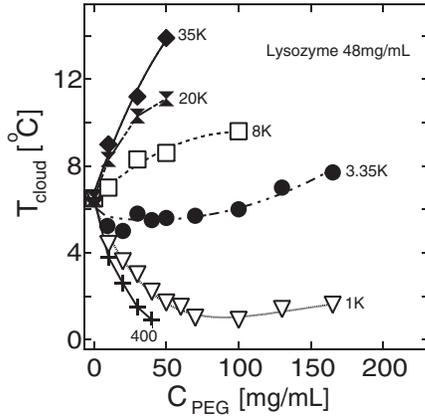
FIG. 1. Cloud point temperatures (T_{cloud}) of lysozyme-PEG mixtures as a function of PEG concentration for different PEG molecular weights ranging from 400 to 35k g/mol at a fixed lysozyme concentration of 48 mg/mL. The lines are guides to the eye.

trations near lysozyme's critical point showed that $\lim_{\rho_2 \to 0} \partial T / \partial \rho_2$ 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. PEG 1k and 8k second virial coefficients as functions of temperature were found to be $B_{22}^{P1k}(T) \, [\mathrm{mL\,mol\,g^{-2}}] = 0.01 - (8.8 \times 10^{-5} \times T[^{\circ}C])$ and $B_{22}^{P8k}(T) \, [\mathrm{mL\,mol\,g^{-2}}] = 5.6 \times 10^{-3} - (9.5 \times 10^{-5} \times T[^{\circ}C])$, which agree well with previous results [12]. Table I 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: $B_{11}(T = 20\,^{\circ}C) = -3.95 \pm 0.3 \times 10^{-4}$ mL mol g^{-1}, $\partial B_{11}/\partial T = 1 \times 10^{-5}$ mL mol g^{-2} $^{\circ}$C^{-1}, and $M_1 = 13\,800 \pm 500$ g mol^{-1}.

Equation (5) 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 [21]. If interference between the scattering from lysozyme and PEG was negligible then $\partial \alpha / \partial c_2 = 0$, as seen from Eq. (5) with $(M_2 n_2)/(M_1 n_1) = 0$. However, Fig. 2(a) shows that $\partial \alpha / \partial c_2 > 0$ consistent with previous work [21]. This demonstrates that interference from lysozyme and PEG scattering may not be ignored as done by Kulkarni *et al.* [22] and that α may not be treated as a constant. Figure 2(b) displays the variation of β with PEG concentration. The solid lines through the data points are the linear fits used to obtain the values of C_{112} by Eq. (6) 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 [23]. The measured dependencies of T_{cloud} and β on PEG concentration show that third virial coefficients must be included in the free energy of lysozyme-PEG mixtures $C_{112} \neq 0$. If $C_{112} = 0$ then Eq. (6) 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. (4) predicts that $\partial T_{sp}/\partial \rho_2$ cannot change sign as a function of PEG molecular weight, which is inconsistent with Fig. 1.

The lysozyme-PEG mixed virial coefficients shown in Table I 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) and (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 g^{-3} 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 g^{-3}. We conclude that adding PEG weakens the attraction between lysozyme molecules in contradiction to depletion theory.

We find experimentally $\lim_{c_2 \to 0} \partial T_{cloud}/\partial c_2 = -0.15$, $0.06 \pm 0.02\,^{\circ}C\,mg^{-1}\,mL^{-1}$ for PEG 1k and 8k, respectively. Using Eq. (4) with the measured virial coefficients obtained independently from SLS yields

TABLE I. PEG molecular weights (M_2) and second virial coefficients (B_{22}) taken from light scattering data at $T = 30\,^{\circ}C$, 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. (5) and (6), 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) and (3).

	M_2 [10^3 g/mol]	N	B_{22} [10^{-3} mL mol/g^2]	B_{22}/B_{22}^{HS}	B_{12} [10^{-4} mL mol/g^2]	B_{12}/B_{12}^{HS}	C_{112} [10^{-4} mL^2 mol/g^3]	C_{112}/C_{112}^{HS}
PEG 1k	0.97 ± 0.1	22	9.2 ± 1.0	0.5	12.9 ± 1.2	0.34	16.1 ± 1.3	0.23
PEG 8k	10.4 ± 0.5	236	3.35 ± 0.12	0.4	1.7 ± 0.3	0.089	3.13 ± 0.2	0.051
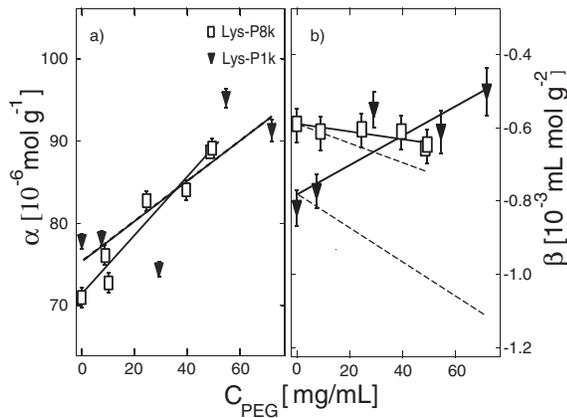
FIG. 2.   The variation of α and β on PEG concentration at $T =$ 30 °C is shown for lysozyme-PEG 1k and PEG 8k mixtures. The solid lines in panel (a) indicate the fits to α used to obtain B_{12} by Eq. (5). The solid lines in panel (b) indicate the fits to β used to obtain C_{112} by Eq. (6). The dashed lines in panel (b) show the dependence of β on PEG concentration assuming $C_{112} = 0$.

$\lim_{c_2 \to 0} \partial T_{sp} / \partial c_2 = -0.03 \pm 0.05$, 0.005 ± 0.008 °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 $\lim_{\rho_2 \to 0} \partial T_{cloud} / \partial \rho_2$. As discussed previously, $\gamma > 0$ for a hard sphere mixture, whereas Fig. 1 shows that the sign of γ 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. (4) do so qualitatively.

This Letter demonstrates that the depletion theory does not describe PEG-lysozyme mixtures. First, 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. Second, 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 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 [24]. 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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