

Supplementary Material: Charge-Controlled Metastable Liquid-Liquid Phase Separation in Protein Solutions as a Universal Pathway Towards Crystallization

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Here we provide supplementary material for the paper Zhang et al., Metastable Liquid-Liquid Phase Separation in Protein Solutions as a Universal Pathway Towards Crystallization, 2011. First, we assemble supporting experimental information. Second, we give a theoretical consideration on the criterion of the second virial coefficient related to the LLPS.

Supporting theoretical information

Effective one-component systems

We briefly elaborate on the phenomenon of a liquid-liquid phase separation in a potentially complex mixture of several components like colloids or proteins in solution, which consists of the colloids/proteins, the solvent, added salt, etc. We provide a simple and intuitively transparent argument how it relates to the second virial coefficient. Often it is natural to focus on the behavior of the largest component, the colloids or proteins, and treating the remaining parts of the mixture as a background medium. This can be done in a rigorous way by mapping the Hamiltonian of the mixture onto an effective one-component system^{4,5} by integrating out the degrees of freedom of those components that should no longer be considered. This mapping changes the interactions in the system from the *bare* interactions between all possible species combination, i.e. protein-water, protein-ion, etc., to *effective* interactions between particles of the remaining species.

For such a system to undergo a phase separation into a low density fluid phase (gas) and a high density fluid phase (liquid), it is important that the effective interaction possesses in addition to the repulsion at short distances an attractive tail. The reason is simple to understand: When a low density phase is transformed into a high density phase, then the system loses entropy, which has to be compensated by the gain in interaction energy due to the attractive tail. This argument holds independent of the precise nature of the effective interaction.

Clearly, the attraction has to be sufficiently strong for a phase separation to occur. A convenient measure for the strength of the attraction is the *effective* second virial coefficient,

which is defined by

$$B_2 = 2\pi \int_0^{\infty} dr r^2 \{1 - \exp(-\beta V_{\text{eff}}(r))\}. \quad (1)$$

If B_2 is positive, the interaction is mainly repulsive and the system behaves hard-sphere-like, if it is negative, then the attraction is strong.

The question is how strong the *effective* interaction has to be in order to drive a phase separation. To this end Vliegenhart and Lekkerkerker made the interesting observation that for various systems the value of the second virial coefficient B_2 at the critical point seems universal⁶:

$$\frac{B_2}{B_2^{HS}} \approx -1.5, \quad (2)$$

with $B_2^{HS} = 16\pi R^3/3$ the second virial coefficient of a hard sphere of radius R . This observation was also confirmed by Noro and Frenkel⁷ and was tested for hard-sphere mixtures⁸ using the depletion potential⁵.

The sticky hard-sphere model was introduced by Baxter⁹ as an example of a system with hard-core repulsion and additional attraction, which can undergo fluid-vapor phase separation and which can be treated analytically. The interaction

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potential is given by

$$\beta V_{\text{eff}}(r) = \begin{cases} \infty & r < \sigma = 2R, \\ -\beta u_0 = \ln\left(\frac{12\tau\Delta}{\sigma+\Delta}\right) & \sigma < r < \sigma + \Delta, \\ 0 & r > \sigma + \Delta, \end{cases} \quad (3)$$

where usually the limit $\Delta \rightarrow 0$ is taken. In this limit one finds for the reduced second virial coefficient

$$\lim_{\Delta \rightarrow 0} \frac{B_2}{B_2^{HS}} = 1 - \frac{1}{4\tau}. \quad (4)$$

Baxter found⁹ that within the Percus-Yevick closure relation at the critical point the stickiness parameter is given by

$$\tau_c = \frac{2 - \sqrt{2}}{6} \approx 0.0976. \quad (5)$$

If the value of the stickiness parameter at the critical point, τ_c is inputted into the expression for the reduced second virial coefficient, Eq. (4), one finds

$$\frac{B_2(\tau = \tau_c)}{B_2^{HS}} = 1 - \frac{1}{4\tau_c} \approx -1.56, \quad (6)$$

which agrees nicely with the aforementioned criterion for the second virial coefficient.

In order to better understand the observation by Vliegenhart and Lekkerkerker we consider phase coexistence between a low and a high density fluid in more detail. A low density fluid phase with density ρ_I can coexist with a high density phase with density ρ_{II} , if they are in mechanical and in chemical equilibrium, i.e.

$$p(\rho_I) = p(\rho_{II}), \quad \text{and} \quad \mu(\rho_I) = \mu(\rho_{II}), \quad (7)$$

where p is the pressure and μ the chemical potential. For mechanical equilibrium to be possible, the pressure at coexistence has to be low, because the pressure of a low density phase will always be low. Therefore, at coexistence, it is possible to expand the pressure into a virial expansion and truncate the series after a few terms with the most prominent contribution being the second virial term:

$$\beta p(\rho) \approx \rho + B_2\rho^2 + B_3\rho^3 + \dots \quad (8)$$

Only, if the attraction is sufficiently strong can the pressure of a high density phase be equally low as that of the low density phase. The onset of a fluid-fluid phase separation is a critical point.

The location of the critical point follows from

$$\left. \frac{\partial p(\rho)}{\partial \rho} \right|_{\rho=\rho_c} = 0 = \left. \frac{\partial^2 p(\rho)}{\partial \rho^2} \right|_{\rho=\rho_c}. \quad (9)$$

Note that the vanishing of the first derivative of the pressure p w.r.t. density ρ expresses the condition of vanishing inverse compressibility or the bulk modulus. This is important because the structure factor $S(q)$ in the limit of $q \rightarrow 0$ reduces to the compressibility χ_T , i.e. $S(q \rightarrow 0) = k_B T \rho \chi_T$. This means that at the critical point or at the spinodal line, when the derivative of the pressure w.r.t. density, or the inverse compressibility, vanishes, the structure factor diverges for $q \rightarrow 0$. If one considers a system at a coexisting density, i.e. on or close to the binodal line, then the state is close to the spinodal, which implies that the compressibility is large, but does not diverge, which can be seen by a large value of $S(q \rightarrow 0)$. The closer the system is to the critical point, the closer the binodal and spinodal lines are. This means that close to the critical point, on the binodal (at either the low or the high coexisting density), the compressibility and hence $S(q \rightarrow 0)$ are large, while further away from the critical point, the difference in density between the binodal and spinodal increases, which means that the compressibility at a coexisting density and the structure factor $S(q \rightarrow 0)$ decrease.

By combining Eqs. (8) and (9) we obtain a set of equations which can be solved e.g. for the second and third virial coefficient at the critical point. Here only the result for B_2 is of interest. One finds that

$$\frac{B_2}{B_2^{HS}} \approx -\frac{1}{4\eta_c} \approx -2.06, \quad (10)$$

with the packing fraction at the critical point due to Baxter⁹

$$\eta_c = \frac{3\sqrt{2}-4}{2} \approx 0.1213. \quad (11)$$

While the agreement between Eqs. (6) and (10) is clearly far from perfect, this simple argument helps to rationalize the origin of the B_2 criterion based solely on the idea of mechanical equilibrium.

Note that the value of the second virial coefficient at the critical point based either on Eq. (6) or on Eq. (10) change somewhat, if other estimates for the location of the critical point are employed, however, the magnitude remains of the right order.

The conclusion is that for $\tau < \tau_c$ or $B_2/B_2^{HS} < -1.5$ the interaction potential is sufficiently strong to drive a phase separation into a low density and a high density phase.

Supporting experimental information

Sample preparation

Human serum albumin, HSA (A9511) and yttrium chloride were purchased from Sigma-Aldrich and used as received. All samples were prepared at 20 °C. A series of protein solutions with various salt concentrations were prepared by mixing stock solutions. No buffer was used to avoid the effect of other co-ions. The phase transitions were determined by optical transmission. Protein concentrations (c_p) in each phase were determined using UV absorption at a wavelength of 280 nm with a coefficient of 0.51 mL/mg¹. LLPS was determined by optical microscopy. The protein-poor (phase-1) and protein-rich phase (phase-2) are separated by centrifugation. $c_p^{(1)}$ was determined directly by UV absorption, and $c_p^{(2)}$ was calculated from the volume of each phase and the initial protein concentration.

Small-angle X-ray scattering

Small-angle X-ray scattering (SAXS) was performed at beam-line ID2, ESRF (Grenoble, France) at a wavelength of the incident beam of 0.08 nm (16038 eV). A q range of 0.07 to 4.2 nm⁻¹ was covered using a sample to detector distance of 2.0 m. The 2D data were normalized and azimuthally averaged to obtain the intensity profiles on an absolute scale; the solvent background was subtracted. For more detailed information on data reduction and q -resolution calibration see Ref.^{2,3}. In order to determine the salt partitioning after LLPS, sample solutions of phase-1 were measured by SAXS at three incoming energies well below ($E1 = 16038$ eV), near ($E2 = 17032$ eV) and on the absorption edge of yttrium ($E3 = 17038$ eV). The measured data were automatically corrected for the varying detector efficiency and normalized to absolute units, by using water as intensity calibration standard. The remaining difference of intensity integrated over a q range of 2.2 - 4.1 nm⁻¹ gives the fluorescence contributed from Y³⁺, which is proportional to salt concentration (c_s). Using a series of pure salt solutions, the fluorescence intensity $\Delta I(E3 - E1)$ as a function of c_s was calibrated (Figure S2, S3). From $\Delta I(E3 - E1)$ of phase-1 $c_s^{(1)}$ was determined, and $c_s^{(2)}$ was calculated from the volume of each phase and the initial salt concentration.

SAXS form factor determined from dilute protein solutions

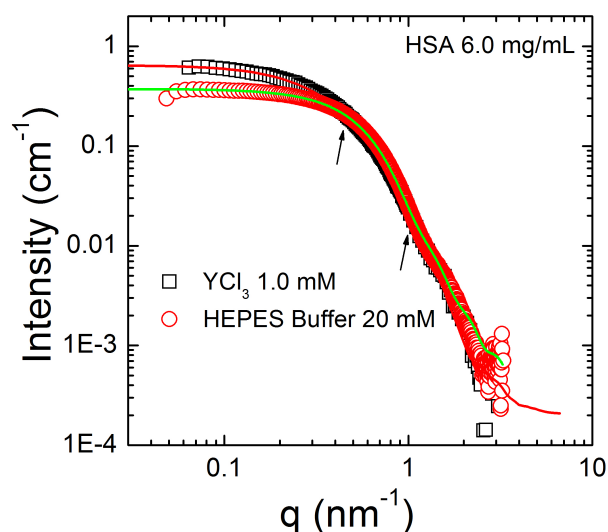


Figure S1: SAXS data with model fitting for HSA 6.0 mg/mL in Regime II, for comparison, SAXS data of HSA with HEPES buffer was also shown.

Calibration of Concentration Measurements of Y³⁺ from X-ray Absorption

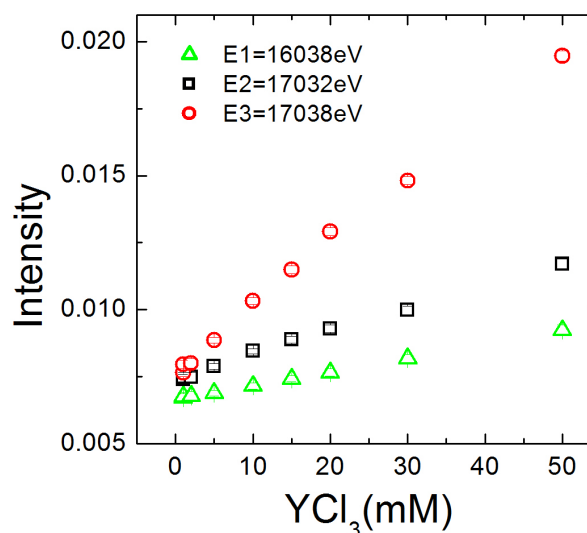


Figure S2: Integrated intensity in the q range of 2.2 to 4.1 nm⁻¹ for pure YCl₃ solutions from 1 to 50 mM at three energies.

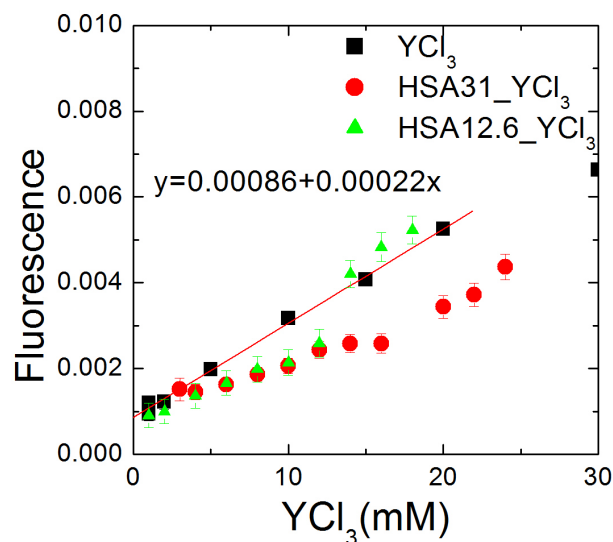


Figure S3: Plots of fluorescence intensity, $\Delta I(E3 - E1)$, as a function of YCl_3 concentration.

References

- 1 D.C. Carter and J.X. Ho, *Adv. Protein Chem.* **45**, 153 (1994).
- 2 T. Narayanan, *Synchrotron small-angle X-ray Scattering*, in: R. Borsali and R. Pecora, *Soft Matter Characterization*, Springer (2008).
- 3 L. Ianeselli, F. Zhang, M.W.A. Skoda, R.M.J. Jacobs, R.A. Martin, S. and Callow, S. Prevost and F. Schreiber, *J. Phys. Chem. B* **114**, 3776 (2010).
- 4 W. G. McMillan and J. E. Mayer, *J. Chem. Phys.* **13**, 276 (1945).
- 5 R. Roth, R. Evans and A. A. Louis, *Phys. Rev. E* **64**, 051202 (2001).
- 6 G. A. Vliegthart and H. N. W. Lekkerkerker, *J. Chem. Phys.* **112**, 5364 (2000).
- 7 M. G. Noro and D. Frenkel, *J. Chem. Phys.* **113**, 2941 (2000).
- 8 M. Dijkstra, R. van Roij, and R. Evans, *Phys. Rev. E* **59**, 5744 (1999).
- 9 R. J. Baxter, *J. Chem. Phys.* **49**, 2770 (1968).