

Crystallization of globular proteins

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Globular proteins can be crystallized by the addition of electrolyte or nonadsorbing polymer. Too much additive, however, gives rise to amorphous precipitates. By comparing existing data on various proteins with previous work on a model colloid-polymer mixture, we suggest an explanation for noncrystallization in terms of a hidden gas-liquid binodal inside the equilibrium fluid-crystal region of the phase diagram. [S1063-651X(97)15802-0]

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I. INTRODUCTION

The crystallization of a protein from solution is the first step in the determination of its structure by x-ray diffraction [1]. Crystallization is frequently brought about by the addition of electrolyte or of nonadsorbing polymer such as polyethylene glycol (PEG). In either case, a short-range intermolecular attraction is induced by the added component.

Recent studies of various globular proteins and related macromolecules [2,3] have shown that crystals are only formed when the second virial coefficient of the induced short-range intermolecular attraction, B_2 , becomes sufficiently negative. Following Rosenbaum, Zamora, and Zukoski [3], values of B_2 for a variety of short-range potentials can be reported by mapping onto the Baxter sticky hard sphere (SHS) model [4], in which the interaction between two spherical particles, diameter σ , is given in terms of a stickiness parameter τ ,

$$\frac{U_{SHS}}{k_B T} = \lim_{\delta \rightarrow 0} \begin{cases} \infty, & r < \sigma \\ \ln \left[\frac{12\tau\delta}{\sigma + \delta} \right], & \sigma < r < \sigma + \delta \\ 0, & r > \sigma + \delta. \end{cases} \quad (1)$$

While $U_{SHS} \rightarrow -\infty$ in the limit $\delta \rightarrow 0$, the second virial coefficient of the Baxter potential remains finite and is given by

$$B_2^{SHS} = 2\pi \int_0^\infty (1 - e^{-U/k_B T}) r^2 dr = \frac{2}{3} \pi \sigma^3 \left(1 - \frac{1}{4\tau} \right). \quad (2)$$

The combined data of George and Wilson [2] and Rosenbaum, Zamora, and Zukoski [3] show that more than 20 globular proteins and related macromolecules have a common crystallization boundary in the τ - ϕ plane, where ϕ is the volume fraction of protein molecules, Fig. 1. Both groups also drew attention to the well-known observation that too much additive, or, equivalently, too negative a B_2 , would lead to amorphous precipitates. No explanation, however, was given for this rather mysterious but important aspect of the art of protein crystallization.

In this paper we give a possible theoretical explanation of the observed cessation of crystallization at large, negative

values of B_2 by drawing a parallel with our recent work [5-7] on a well-characterized model colloid-polymer mixture.

II. MODEL COLLOID-POLYMER MIXTURE

Our system consists of sterically stabilized polymethylmethacrylate (PMMA) particles and random-coil polystyrene (PS) dispersed in *cis*-decalin. Observations were carried out at room temperature, where *cis*-decalin is a slightly better than theta solvent for PS [8]; i.e., the polymer coils are effectively interpenetrable as far as their mutual interaction is concerned. Previous work [9] suggests that the particles interact as nearly perfect hard spheres; the polymer appears to be nonadsorbing [5] and interacts with the particles merely by excluded volume. The exclusion of polymer from the re-

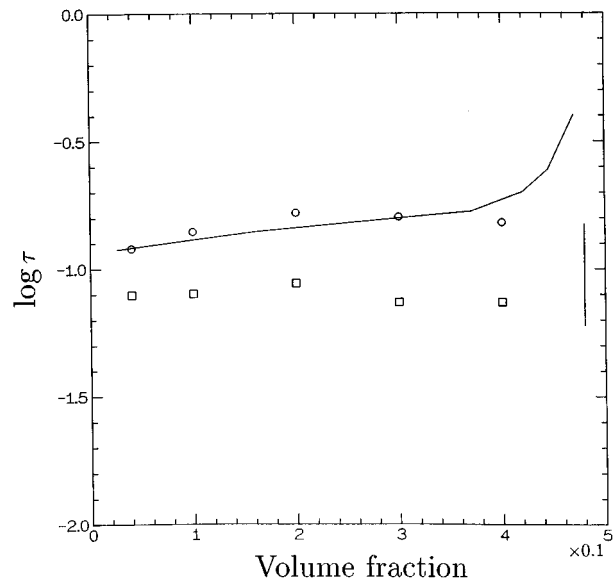


FIG. 1. Phase diagram for particles with short-range attraction. Line, common crystallization boundary for the four macromolecules in Rosenbaum, Zamora, and Zukoski [3]. The crystallization boundaries of globular proteins studied by George and Wilson [2] also fall closely around this curve. O, crystallization boundary for the colloid-polymer mixture in [5]; □, noncrystallization boundary for the same system [7]. The vertical bar gives $0.06 < \tau < 0.15$, the optimal range for protein crystallization identified in Ref. [3].

gion between two nearby particles gives rise to an unbalanced osmotic pressure pushing the particles together, resulting in an attractive depletion potential $U_{\text{dep}}(r)$, which can lead to phase separation. The range and strength of U_{dep} can be separately tuned by altering the polymer molecular weight (and therefore coil size) and concentration, respectively. The phase behavior depends on the polymer to particle size ratio $\xi = r_g/R$. Small ξ gives an expanded region of colloidal fluid-crystal coexistence [5]. For larger ξ , colloidal gas-liquid coexistence becomes possible [6].

The experimental boundary between single-phase fluid and crystal-fluid coexistence regions for $\xi = 0.08$, previously given [5] in terms of the colloid volume fraction (ϕ) and the polymer weight concentration, is mapped onto the ϕ - τ plane in Fig. 1 [with B_2 calculated from $U_{\text{dep}}(r)$ using equations in [6]]. The deviation of our data from the average crystallization boundary taken from Ref. [3], $\tau \sim 0.15$ for $0 \leq \phi \leq 0.4$, is within the spread of the two data sets. This gives us confidence that the same fundamental physics underlies our system and the diverse globular protein solutions investigated previously in [2] and [3].

III. CESSATION OF CRYSTALLIZATION

The position of the crystallization boundary in our colloid-polymer mixture is well accounted for by a simple statistical mechanical theory [10,5]. In the theory, polymer molecules are treated as an ideal gas (i.e., points, a reasonable approximation for the near- θ conditions used in our experiments), and the colloids are hard spheres of radius R (to model the interaction between sterically stabilized PMMA spheres). However, a polymer molecule is excluded from coming closer than a distance δ to the surface of a colloidal particle (a simple model of the excluded volume interaction between polymer and particle). In practice we expect $\delta \sim r_g$. The phase behavior of this nonadditive hard sphere model is then solved within a mean-field framework, which predicts that the topology of the phase diagram depends on the ratio δ/R .

For $\delta/R = 0.08$, this theory predicts crystallization for all polymer concentrations across a crystal-fluid boundary at $\tau \leq 0.15$, which matches well the observed crystallization boundary in our experimental system, Fig. 1. In the experiments, however, crystallization was suppressed at high enough polymer concentrations (or, equivalently, at τ values sufficiently lower than ~ 0.15) [5,7]. Beyond an experimentally reproducible *noncrystallization boundary*, Fig. 1, various types of nonequilibrium aggregation behavior were observed, culminating, at the highest polymer concentrations (or smallest τ), in the formation of transient gels [7]. Extensive small-angle light scattering investigations of our model colloid-polymer mixture [7] suggest that the noncrystallization boundary should be identified with a *metastable gas-liquid binodal* buried in the equilibrium fluid-crystal coexistence region, Fig. 2. The presence of this boundary can be understood as follows.

Following our earlier work [10] we write the free energy F of a colloid-polymer mixture as a function of ϕ , the colloid volume fraction, and μ_p , the polymer chemical potential [11], $F = F(\phi, \mu_p)$. F can be calculated within a mean-field framework for a disordered arrangement of colloids and

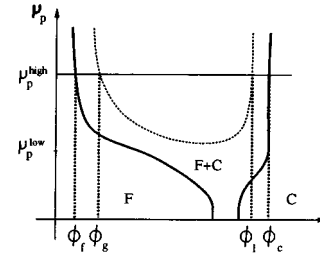


FIG. 2. Schematic phase diagram of a colloid-polymer mixture in which the ratio of the polymer to colloid size is ≤ 0.25 . The variables are the colloid volume fraction ϕ and the polymer chemical potential μ_p . A sample in the F (C) region remains a single-phase colloidal fluid (crystal). The equilibrium phase boundary is in bold. Above this boundary, in the $F+C$ region, a sample should separate into coexisting colloidal fluid and crystal phases. Above the dotted curve, which is the metastable gas-liquid binodal, however, the growth of colloidal crystals is disrupted, and various non-equilibrium aggregation behaviors are observed. The free energy diagrams associated with the two indicated polymer chemical potentials μ_p^{low} and μ_p^{high} are shown in Figs. 3(a) and 3(b), respectively.

polymers, the fluid branch, and an ordered (face-centered-cubic) arrangement of colloids with polymers randomly dispersed, the crystal branch. At low polymer chemical potentials, the fluid and crystal branches each show a single minimum, Fig. 3(a). This gives rise to single-phase fluid, fluid-crystal coexistence, or single-phase crystal. The colloid concentrations in coexisting fluid and crystal phases are obtained by the common tangent construction (see, e.g., [12]).

At higher polymer chemical potentials, however, the fluid branch of the free energy shows a double minimum structure, Fig. 3(b). The points on the *equilibrium* phase boundary at this polymer chemical potential, ϕ_f and ϕ_c (cf. Fig. 2), are still obtained by constructing the (lowest) common tangent between the fluid and crystal branches. However, one could easily discard the crystal branch and perform the double tangent construction on the fluid branch of the free energy alone. In this way, a gas-liquid binodal is obtained, which lies at ϕ_g and ϕ_l at this polymer chemical potential; compare Figs. 2 and 3(b). This metastable gas-liquid binodal is buried

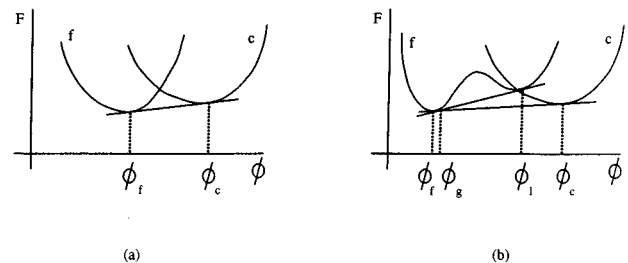


FIG. 3. Schematic free energy diagrams $F(\phi, \mu_p)$. (a) Low polymer chemical potential μ_p^{low} (see the vertical axis in Fig. 2). Any homogeneous colloidal fluid with a volume fraction between ϕ_f and ϕ_c will phase separate into coexisting fluid and crystal phases with those volume fractions (the common tangent construction). (b) At high polymer chemical potential μ_p^{high} , the fluid branch has a double minimum structure. The common tangent construction can be used to trace out both the equilibrium phase boundary ϕ_f and ϕ_c , and the metastable gas-liquid binodal ϕ_g and ϕ_l .

within the equilibrium fluid-crystal coexistence region in the phase diagram, Fig. 2 [13]. The position of the gas-liquid binodal predicted by our mean-field theory [10] corresponds well with the experimental noncrystallization boundary found in our colloid-polymer mixture [7].

Our noncrystallization boundary lies very close to the $\tau \sim 0.06$ boundary identified by Rosenbaum, Zamora, and Zukoski beyond which proteins will not crystallize. We therefore suggest that the failure of proteins to crystallize is also due to a hidden gas-liquid binodal. The double-well structure in the fluid branch of the free energy at low τ favors local density fluctuations which produce amorphous structures. The metastable liquid-gas spinodal for the Baxter SHS model was plotted in Fig. 1 of [3], buried inside the crystallization region. This spinodal (and thus the associated binodal) lies at values of τ a little higher than the noncrystallization boundary at $\tau \sim 0.06$. Note, however, that the precise location of the equilibrium crystal-fluid and metastable gas-liquid boundaries for the SHS system are still uncertain. Moreover, Stell warned that the procedure of mapping to the SHS model via B_2 is likely to be increasingly invalid for values of τ less than unity [14].

IV. CONCLUSION

Based on a comparison with existing work on a model colloid-polymer mixture, we have associated the failure of globular proteins with large, short-range intermolecular attractions to crystallize with the presence of a hidden gas-liquid binodal in the phase diagram. Indeed, in complete analogy to the case of colloid-polymer mixtures, metastable, amorphous precipitates are formed rather than crystals (which is the prediction of equilibrium thermodynamics)

when the noncrystallization boundary, $\tau \sim 0.06$, is crossed.

The practice of relating nonequilibrium behavior (in this case the failure to crystallize) to hidden, metastable phase boundaries within equilibrium regions of two-phase coexistence is a common one in metallurgy. The possibility of such a boundary was pointed out explicitly by Cahn in an early review [15] (see also Sec. 10.1.6 in [12]), and forms the basis of a metallurgical rule of thumb — the Ostwald rule of stages [16]. The presence of such a metastable phase boundary inside an equilibrium two-phase region is related to the triple-minima structure of the free energy, Fig. 3(b). The lowest two minima give rise to two coexisting equilibrium phases (via the common tangent construction). The third, metastable, minimum, though of no significance for equilibrium phase behavior, nevertheless can decisively influence phase transition *kinetics*, as demonstrated by the cessation of crystallization in the systems discussed in this paper. The theoretical basis of such effects is the subject of active investigation [17].

Note added in proof: Recently, two publications have appeared that throw further light on the issues discussed here [18,19].

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- [1] P. C. Weber, *Adv. Protein Chem.* **41**, 1 (1991).
 [2] A. George and W. Wilson, *Acta. Crystallogr. D* **50**, 361 (1994).
 [3] D. Rosenbaum, P. C. Zamora, and C. F. Zukoski, *Phys. Rev. Lett.* **76**, 150 (1996).
 [4] R. J. Baxter, *J. Chem. Phys.* **49**, 2770 (1968).
 [5] W. C. K. Poon, J. S. Selfe, M. B. Robertson, S. M. Ilett, A. D. Pirie, and P. N. Pusey, *J. Phys. (France) II* **3**, 1075 (1993).
 [6] S. M. Ilett, A. Orrock, W. C. K. Poon, and P. N. Pusey, *Phys. Rev. E* **51**, 1344 (1995).
 [7] W. C. K. Poon, A. D. Pirie, and P. N. Pusey, *Faraday Discuss.* **101**, 65 (1995).
 [8] G. C. Berry, *J. Chem. Phys.* **44**, 4550 (1966).
 [9] See, e.g., S. M. Underwood, J. R. Taylor, and W. van Meegen, *Langmuir* **10**, 355 (1994).
 [10] H. N. W. Lekkerkerker, W. C. K. Poon, P. N. Pusey, A. Stroobants, and P. B. Warren, *Europhys. Lett.* **20**, 559 (1992).
 [11] The chemical potential μ_p , which determines the polymer fugacity, must be equal in any coexisting phases. Using this variable reduces the number of equations to be solved in calculating the phase diagram. See [10] for the relation between μ_p and the polymer concentration.
 [12] R. T. DeHoff, *Thermodynamics in Material Science* (McGraw-Hill, New York, 1992).
 [13] At larger polymer to colloid size ratios, this double minimum can give rise to a region of colloidal gas-liquid coexistence in the equilibrium phase diagram [10,6].
 [14] G. Stell, *J. Stat. Phys.* **63**, 1203 (1991).
 [15] J. W. Cahn, *Transc. Metall. Soc. AIME* **242**, 166 (1968), Fig. 6. In particular, compare Figs. 6(c) and 6(b) with Figs. 2(b) and 2(c) of this paper.
 [16] This rule states that the first phase to emerge in a quench experiment is often not the thermodynamically stable phase, but rather a metastable one. See W. Ostwald, *Z. Phys. Chem.* **22**, 289 (1897).
 [17] For a nonconserved order parameter, see J. Bechhoefer, H. Löwen, and L. S. Tuckerman, *Phys. Rev. Lett.* **67**, 1266 (1991); for a conserved order parameter, see R. M. L. Evans, W. C. K. Poon, and M. E. Cates (unpublished).
 [18] M. L. Broide, T. M. Tominic, and M. D. Saxowsky, *Phys. Rev. E* **53**, 6325 (1996).
 [19] N. Ashene, A. Lomakin, and G. B. Benedek, *Phys. Rev. Lett.* **77**, 4832 (1996).