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Self-crowding induced phase separation in protein dispersions

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The coupling between protein conformation, molecular volume, and solution phase behaviour is studied theoretically for a two-state, coarse-grained protein model in which protein molecules can reversibly switch between a native and a non-native excited state. In the model, native and non-native conformers are represented by perfect spheres with different hard-core diameters. We presume the larger, non-native species to attract each other through some unspecified potential. We find that at low concentrations the native state is stabilised energetically and that at high concentrations the native state is again stabilised but this time by self-crowding, i.e., a lack of free volume. These two regimes are separated by two first-order transitions from a region where the non-native conformational state is prevalent, stabilised by attractive interactions between the proteins. The calculated phase diagram is very sensitive to even quite small differences in particle volumes and has unusual features, including the loss of a critical point if the size difference is sufficiently large. © *2015 AIP Publishing LLC.* [http://dx.doi.org/10.1063/1.4922927]

I. INTRODUCTION

Theoretical models of protein phase behaviour in aqueous solution often presume the proteins to be structureless, undeformable, and perfectly spherical. However, even if approximately spherical, proteins do have an internal (folded) structure that could respond to changes in their local environment. This includes changes in physico-chemical solution conditions,[1] binding to other proteins,[2–5] and competition for water at elevated protein concentrations.[6] The response of the structure of protein molecules to such changes in local environment can affect the way they interact with other proteins in the solution, e.g., because their effective volume, which includes tightly bound hydration water, changes. This implies that in crowded environments, where the free volume is limited due to the presence of a high concentration of macromolecules, such as is the case in the cell,[7,8] interactions between proteins may be different from that in a dilute solution.[9–13] Indeed, it is well known that crowding tends to increase protein complexation rates and shifts binding equilibria towards assembled states in order to increase the free volume available in the solution.[9] Crowding stabilises the compact (native) protein conformation for the same reason.[6,14–16]

How precisely crowding influences protein conformation and how this in turn influences the phase and aggregation behaviour remain rather poorly understood, despite a considerable research effort in both fields.[9,17] This is remarkable because applications in the food[18] and pharmaceutics industry[19] often involve dense protein formulations that are difficult to process and that exhibit undesirable processes such as syneresis, i.e., the expulsion of water. Still, that crowding, including self-crowding, is important and that the study of proteins in dilute solutions is not necessarily helpful in understanding the physical properties of such systems is well recognised.[20] Indeed, in an increasing number of experiments, crowding agents are added or the experiments are carried out in living cells to address crowding-related effects, e.g., in complexation and folding.[21]

While our understanding of how crowding induces phase separation in multi-component systems is relatively advanced,[22–24] much less is known about how self-crowding affects protein phase behaviour in the liquid phase. Here, we study theoretically how a combination of volume exclusion and attractive interactions influences the switching between two conformational states of a model protein and through that its solution phase behaviour. We show that if these two conformational states have different effective volumes this profoundly affects the phase diagram, even if this difference is very small. We find that under appropriate conditions, increasing the concentration of proteins first liquid-liquid phase separation occurs in which in the dense phase the non-native state, presumed larger than the native state, is more prevalent. A second first-order phase transition occurs at higher concentrations, in which the (smaller) native state becomes more stable again because of its lower volume exclusion. The location of the two two-phase domains in the phase diagram turns out to be very sensitive to the size ratio of the native and non-native protein states. For large enough size difference, these domains meet in a single point that is not a critical point.

In the remainder of this paper, we present our model free energy in Sec. II and use this to determine the equilibrium phase behaviour in Secs. III and IV. In Sec. III, we investigate how the equilibrium fraction of proteins in the non-native state depends on the concentration and on the model parameters, and show that there are two first-order conformational phase transitions. This leads to a total of 5 qualitatively different relationships between protein concentration and conformation. In Sec. IV, we determine the corresponding phase diagrams and show how both first-order conformational phase transitions are coupled to liquid-liquid phase separation. Furthermore,

we show that a distinction between two qualitatively different types of phase diagram can be made. Finally, in Sec. V we summarise and discuss the results.

II. FREE ENERGY MODEL

Our model dispersion consists of an implicit solvent and two types of spherical particle that we denote 1 and 2, with diameters d_1 and $d_2 \geq d_1$. Particles 1 and 2 represent the two different states of our protein and can reversibly inter-convert where a free energy penalty $\epsilon \geq 0$ is assigned to particle 2 that we identify as the non-native state. Both types of particle interact via a hard-core volume exclusion, whereas particles in state 2 also attract each other via some unspecified interaction potential. We construct a free energy function for this model by combining the free energy of a bidisperse hard-sphere fluid that we derive from the polydisperse Boublik-Mansoori-Carnahan-Starling-Leland (BMCSL) equation of state,[25,26] a van der Waals-like contribution that accounts for attractive interactions between the non-native species of protein and terms describing the "chemical" equilibrium between the two states.[27]

The BMCSL equation of state is widely used and has been described as both simple and accurate.[28] We realise that more sophisticated and accurate approaches such as fundamental measure theory have been put forward.[28,29] However, as we have shown in previous work for a monodisperse model protein dispersion,[27] qualitatively the predicted phase behaviour is insensitive to different thermodynamic models and because of this we believe the use of the simpler BMCSL equation of state is justified.

The BMCSL equation of state can be integrated isothermally over the volume to obtain the corresponding Helmholtz free energy. If we define the dimensionless free energy density as $f = v_1 \beta F/V$, where v_1 is the volume of a single protein that is in its native state, $1/\beta = k_B T$ is the thermal energy and V is the system volume, then the BMCSL contribution to the free energy reads

$$f^{\mathrm{HS}} = (1-\eta)\,\phi \ln(1-\eta)\,\phi + \frac{\eta\phi}{\kappa^3}\ln\frac{\eta\phi}{\kappa^3} - (1-\eta)\,\phi - \frac{\eta\phi}{\kappa^3} + \phi\left(1 + \frac{1-\kappa^3}{\kappa^3}\eta\right) \times \left[\frac{3\,(1-y_1-y_2-y_3/3)}{2(1-\phi)^2} - \frac{3\,(1-y_1+y_2+y_3)}{2} + \frac{3y_2+2y_3}{1-\phi} + (y_3-1)\ln(1-\phi)\right], \tag{1}$$

where ϕ is the total protein volume fraction and η is the fraction of the volume of protein that corresponds to proteins in the non-native state. The terms y_1, y_2, and y_3 are defined as

$$y_1 = \eta\,(1-\eta)\,\frac{(1-\kappa)^2\,(1+\kappa)}{\kappa^3 + (1-\kappa^3)\,\eta}, \tag{2}$$

$$y_2 = \eta\,(1-\eta)\,\frac{(1-\kappa)^2\,(\kappa + (1-\kappa)\,\eta)}{\kappa^3 + (1-\kappa^3)\,\eta}, \tag{3}$$

$$y_3 = \frac{(\kappa + (1-\kappa)\,\eta)^3}{\kappa^3 + (1-\kappa^3)\,\eta}, \tag{4}$$

where $\kappa = d_2/d_1$ with d_2 the effective diameter of the protein if in the non-native state and d_1 the effective diameter of the protein in its native state. We presume the native conformation to be the more compact conformation, so $\kappa \geq 1$.

To reduce parameter space and to assure that our model shows the consequences of conformational switching and self-crowding on protein phase behaviour as clearly as possible, we have fixed the strength of attractive interactions involving particles of type 1 at zero and only let particles of type 2 attract each other. Within a mean-field van der Waals-type of approximation,[30] we account for this in a (dimensionless) free energy density of the form

$$f^{\mathrm{vdW}} = -\frac{\chi}{2\kappa^3}(\eta\phi)^2, \tag{5}$$

where χ describes the attractive part of the interaction between particles of type 2 and is given in units of thermal energy ($k_B T$). As we presume particle type 2 to be the high-energy, non-native state of the proteins, we add a free energy,

$$f^{\mathrm{Conf}} = \frac{\eta\phi}{\kappa^3}\epsilon, \tag{6}$$

where κ enters because of our normalisation of the free energy density.

The total dimensionless free energy density of the model two-state protein dispersion is given by the sum

$$f = f^{\mathrm{HS}} + f^{\mathrm{vdW}} + f^{\mathrm{Conf}}, \tag{7}$$

where f^{HS} is given by Eq. (1), f^{vdW} by Eq. (5), and f^{Conf} by Eq. (6). Note that for $\kappa = 1$, we retrieve the dimensionless free energy density for a monodisperse Carnahan-Starling model.[31]

Because our proteins can reversibly switch between conformations of different effective volumes, neither the overall protein volume fraction, ϕ, nor the fraction of the protein volume corresponding to proteins in the non-native state, η, is a conserved quantity. While the quantity η may seem unusual, it is directly related to the number fraction of proteins in the non-native state, η_{N}, via the relation

$$\eta_{\mathrm{N}} = \eta/\left(\kappa^3 + \left(1-\kappa^3\right)\eta\right). \tag{8}$$

We realise that this parameter offers a more intuitive description of the conformational state of the proteins, however, the parameter η enters naturally in our hard-sphere free energy because of the BMCSL equation of state that we invoke.

While the overall protein volume fraction, ϕ, might seem a natural concentration scale, in our case it is somewhat unusual because it is not conserved. The overall number density of

proteins *is* a conserved quantity and it is possible to introduce a dimensionless concentration that is proportional to this quantity and that is also conserved. A natural dimensionless concentration that does that is the volume fraction that the proteins would have if all proteins were in the native state,

$$\phi_{\rm Na} = \phi\left(1 + \frac{1-\kappa^3}{\kappa^3}\eta\right). \tag{9}$$

Importantly, experimentally it is readily determined because it is a direct measure for the amount of proteins in the solution.

In summary, we have put forward a dimensionless free energy density for our model protein dispersion. Solution conditions and protein properties are reflected in the parameters χ, ϵ, and κ, while the thermodynamic state of the dispersion is given by ϕ or $\phi_{\rm Na}$ and η or $\eta_{\rm N}$. In Sec. III, we determine the equilibrium fraction of proteins in the non-native state as a function of concentration, and show that volume exclusion stabilises the native state at high concentrations.

III. STABILIZATION OF THE NATIVE STATE AT HIGH CONCENTRATIONS

The equilibrium fraction of proteins in the non-native state minimises the free energy for a given amount of dissolved protein, $\phi_{\rm Na}$. Hence, to determine the equilibrium fraction of proteins in the non-native state, we set

$$\left(\frac{\partial f}{\partial \eta_{\rm N}}\right)_{\phi_{\rm Na},\chi,\epsilon,\kappa} = 0. \tag{10}$$

We solve the resulting implicit equation numerically and determine whether they are free energy minima or maxima. As we shall see, the local maximum values are physically relevant, because they correspond to a free energy barrier between different (meta-)stable dispersion states with different fractions of proteins in the non-native state at fixed concentration. Furthermore, we will show that these local maxima can be crossed by two different first-order conformational phase transitions at fixed concentrations, giving rise to no fewer than 5 qualitatively different relations between the equilibrium average protein conformation, $\eta_{\rm N}^{\rm eq}$, and protein concentration.

We determine the loci of liquid-liquid phase separation by equating chemical potentials and pressures in the coexisting phases. These can be calculated from our free energy, Eq. (7), where the chemical potential is proportional to $\partial_{\phi_{\rm Na}} f$ and the osmotic pressure to $-f + \phi_{\rm Na}\partial_{\phi_{\rm Na}} f$. In this section, we focus first on how the equilibrium number fraction of proteins in the non-native state, $\eta_{\rm N}^{\rm eq}$, varies with protein concentration, $\phi_{\rm Na}$. In Sec. IV, we discuss the phase diagrams we obtain in more detail.

To set the stage, let us first consider qualitatively how the equilibrium value of the fraction of proteins in the non-native state, $\eta_{\rm N}^{\rm eq}$, depends on the protein concentration, $\phi_{\rm Na}$, and the other three model parameters, χ, ϵ, and κ. This dependence essentially involves four different competing effects. (The first three effects also hold for a system in which proteins of both conformations are of equal size, that is, for $\kappa = 1$.) These are: (1) the entropy of mixing, which favours equal amounts of proteins in both conformations, (2) attractive interactions of strength χ between proteins in the non-native state, which favour as many proteins in the non-native state as possible, and (3) the free energy penalty associated with the non-native state, ϵ ($\geq$0), which favours as few proteins as possible in the non-native state. The competition between the latter two effects is strongly concentration dependent. The size difference between the two conformations introduces a fourth competing effect: (4) self-crowding (excluded-volume) effects that favour as few proteins as possible in the non-native state. This becomes more important with increasing protein concentration, if the system runs out of free volume.

To illustrate how self-crowding influences how $\eta_{\rm N}^{\rm eq}$ varies with $\phi_{\rm Na}$, we set $\chi = 6$ and $\epsilon = 0$, both in units k_BT. We focus on the case where the effective diameter of the protein increases by a mere 5 percent upon transitioning to the non-native state, so $\kappa = 1.05$ and compare this with the case where $\kappa = 1$ and self-crowding is absent. Fig. 1(a) shows that without self-crowding, $\eta_{\rm N}^{\rm eq}$ increases monotonically with concentration. Fig. 1(b), on the other hand, shows that with self-crowding this is no longer the case: self-crowding suppresses the non-native state at high enough concentration. As a result of this, the dependence of $\eta_{\rm N}^{\rm eq}$ on concentration exhibits a maximum. Notice that the area shaded in gray in Fig. 1(b) corresponds to

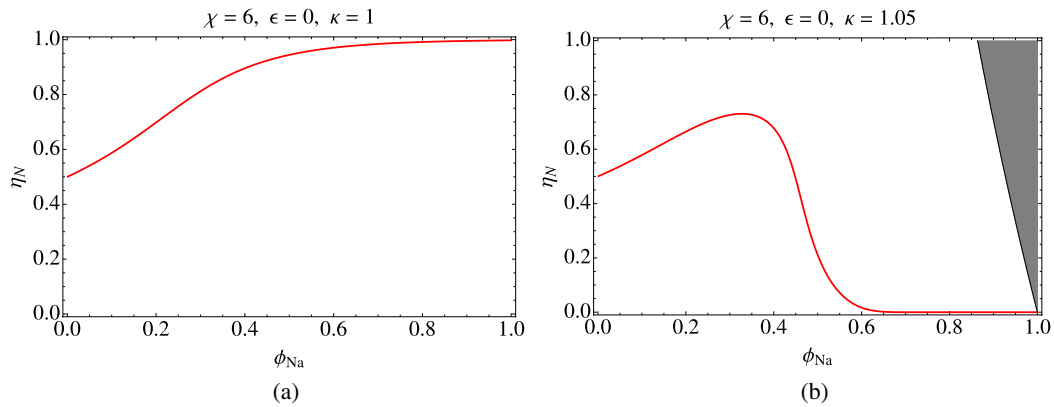

FIG. 1. The equilibrium fraction of proteins in the non-native state, $\eta_{\rm N}^{\rm eq}$, as a function of protein concentration, $\phi_{\rm Na}$. The strength of the attractive interactions between the non-native proteins is $\chi = 6.0\, k_BT$ and the free energy penalty associated with the non-native state is $\epsilon = 0$. (a) Both conformers are of equal size, $\kappa = 1$. (b) The non-native conformer is 5 percent larger than the native conformer, $\kappa = 1.05$. The shaded area indicates unphysical dispersion states where the protein volume fraction exceeds 1.

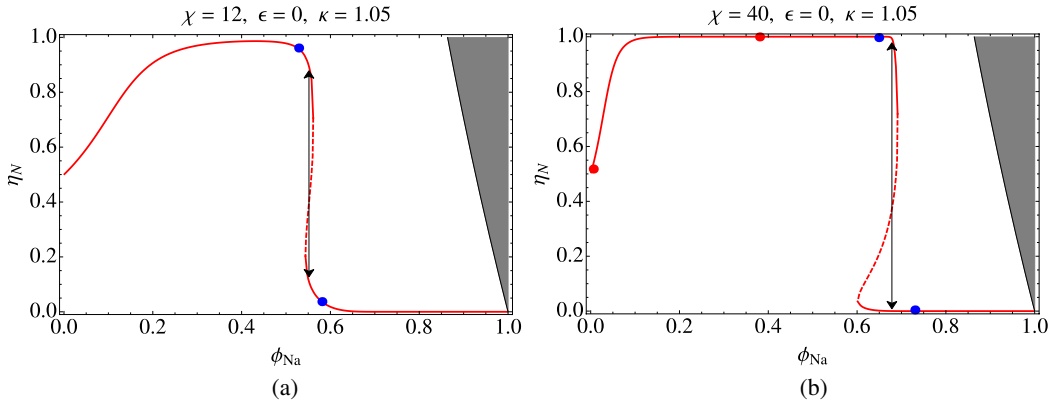

FIG. 2. The equilibrium fraction of proteins in the non-native state, η_N^{eq}, as a function of protein concentration, ϕ_{Na} and a size difference of 5 per cent, $\kappa = 1.05$ and zero free energy difference between the two conformers, for (a) $\chi = 12$ and (b) $\chi = 40$. In both figures, the solid line corresponds to a (local) minimum while the dashed line corresponds to a maximum in the free energy. The concentration at which a first-order conformational phase transition would occur in a homogeneous solution is indicated by a double-pointed arrow. In reality, the solution phase separates and coexisting phases are indicated by the red and blue dots. See also the caption of Fig. 1 for further details.

actual volume fractions larger than unity, which of course is unphysical.

If we increase the strength of the attractive interaction between proteins in the non-native state from $\chi = 6$ to 12 and 40, this leads to a qualitatively different behaviour. Fig. 2(a) shows that for $\chi = 12$ the relation between conformation and concentration exhibits a van der Waals-like loop, signifying a first-order conformational phase transition. The dashed red line in Fig. 2(a) corresponds to a local maximum in the free energy that separates a local (meta-stable) and a global minimum (stable) free energy dispersion state for a fixed value of ϕ_{Na}.

Indicated in the figure (with a double-pointed arrow) is where the conformational transition would occur if there was no macroscopic phase separation. This happens at equal free energies provided the concentration is fixed. In fact, in Sec. IV, we show that this conformational transformation is intimately linked to the transition from a homogeneous dispersion to a liquid-liquid phase separated dispersion in which the concentrations and the average conformational states of the proteins are not equal. The conditions for which we have coexisting phases are indicated in the figure by blue and red dots.

The conformational phase transition that occurs is a result of the competition between self-crowding effects and the attractive interactions between proteins in their non-native state, which respectively favours a state with most proteins in their native and non-native state. With an increase of the strength of the attractive interactions between the proteins in the non-native state, the non-native conformation remains thermodynamically stabilised up to a higher concentration when crowding effects take over, as illustrated in Fig. 2(b).

If the free energy difference between the native and non-native state is increased to a non-zero value, this increases the thermodynamic stability of the native conformation even if proteins in the non-native state strongly attract each other. Fig. 3(a) shows that for $\chi = 12$, $\epsilon = 3$, and $\kappa = 1.05$, the native conformation is thermodynamically stable for all concentrations. The effect of self-crowding becomes clear if we compare this to the situation where both conformations are of equal size and self-crowding plays no role, Fig. 3(b). In this case, the non-native conformation is thermodynamically stabilised at high concentrations.

In Fig. 3(b), a second type of van der Waals-like loop presents itself, different from the one shown in Fig. 2. Associated with it is another first-order conformational phase transition that results from the competition between the tendency of the proteins to remain in their native state due to the increase in

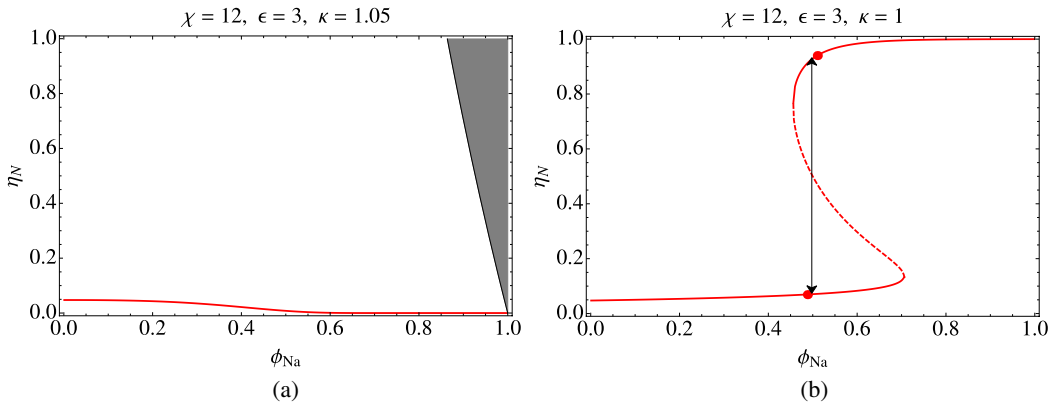

FIG. 3. The equilibrium fraction of proteins in the non-native state, η_N^{eq}, as a function of the protein concentration, ϕ_{Na}. Refer to the caption of Fig. 2 for further details. For attractive interactions of strength $\chi = 12$ and a free energy penalty of $\epsilon = 3$. (a) Size difference $\kappa = 1.05$. (b) No size difference, $\kappa = 1$. The van der Waals-like loop that is shown has a different physical origin from the one shown in Fig. 2(b), see the main text.

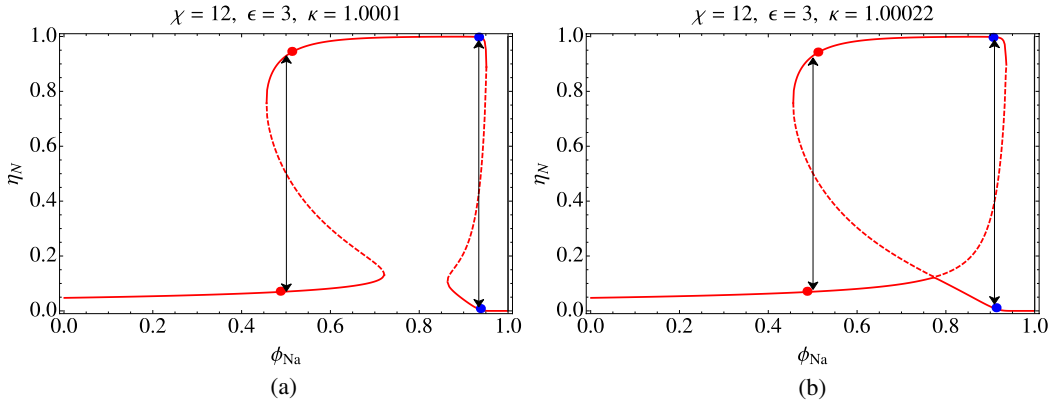

FIG. 4. The equilibrium fraction of proteins in the non-native state, η_N^{eq}, as a function of protein concentration, ϕ_{Na} for attractive interactions of strength $\chi = 12$ and a free energy penalty of $\epsilon = 3$. Refer to Fig. 2 for further details. For a size difference (a) $\kappa = 1.0001$ and (b) $\kappa = 1.0002$.

conformational free energy, ϵ, and the tendency of the proteins to switch to the non-native state and lower the free energy through attractive interactions of strength χ.

Let us now consider how the relation between protein concentration and conformation changes, as we increase the value of κ by a very small amount from 1 up to 1.05, while the other two model parameters remain fixed at $\chi = 12$ and $\epsilon = 3$. See Figures 3(b) and 3(a). If the effective diameter of the non-native state of the protein increases by just 0.01 percent to $\kappa = 1.0001$, a second first-order conformational phase transition appears at high concentration, see also Fig. 4(a). This transition has the same physical origin as the one in Fig. 2, where self-crowding causes the larger non-native state to become unfavourable at high volume fractions. With increasing κ, self-crowding effects become significant at a lower protein concentration, and the van der Waals-like loop at high concentrations shifts to lower concentrations. This continues until $\kappa \approx 1.0002$, when the lower boundary of both loops meets at a single point, see Fig. 4(b).

A further increase in κ leads to a change in the structure of the free energy landscape, where both van der Waals-like loops merge, separating a regime of (meta-)stable dispersion states with proteins mostly in their native state from a regime of (meta-)stable dispersion states with proteins mostly in their native state. See Fig. 5. The dispersion state with most proteins in the non-native state is only stable in between the two first-order conformational phase transitions. Even though the structure appears to be quite different, the two first-order conformational phase transitions that we previously discussed still exist and the behaviour of the proteins remains qualitatively the same.

Increasing κ further enhances self-crowding effects and causes the non-native state to be increasingly unfavourable, and the concentration range over which it is stabilised decreases. This situation remains unchanged up to a critical value of κ at which the two first-order conformational phase transitions merge at a single value of ϕ_{Na}. In Secs. IV and V, we shall see that this corresponds to the "critical point" of two liquid-liquid phase separation transitions. For a further increase in κ, all of the dispersion states with proteins mostly in their non-native state on the upper η_N^{eq} curve become meta-stable, i.e., they have a higher free energy than the corresponding states at lower values of η_N. Increasing κ up to $\kappa = 1.05$ causes these meta-stable states to disappear altogether and the situation as shown in Fig. 3(a) is retrieved.

We have now covered all qualitatively different relationships between protein concentration and equilibrium conformation. These are the result of the four different competing effects discussed at the beginning of this section. In Sec. IV, we investigate in more detail under what circumstances phase separation can occur and if it indeed occurs, what the composition of the coexisting phases is. We shall see that the two types of van der Waals-like loop that we discovered in this section are intimately linked with two consecutive phase separations of the model dispersion that occur with increasing concentration.

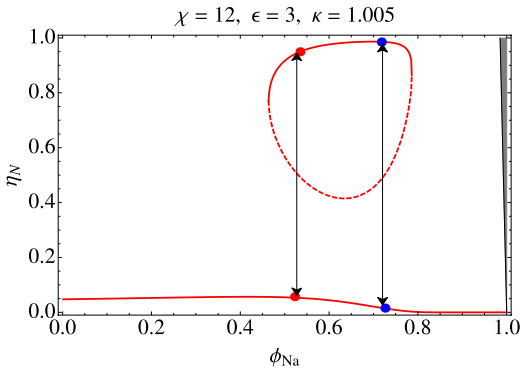

FIG. 5. The equilibrium fraction of proteins in the non-native state, η_N^{eq}, as a function of protein concentration, ϕ_{Na} for $\chi = 12$, $\epsilon = 3$, and $\kappa = 1.005$. See Fig. 2 for further details.

IV. SELF-CROWDING INDUCED PHASE SEPARATION

Phase separation occurs if the solution has a concentration in between the concentrations of two coexisting phases, which are fixed by the physico-chemical conditions of the solution. Under conditions of thermodynamic equilibrium, the temperatures of the co-existing phases must be equal, as must the chemical potential of the proteins and the osmotic pressures. For the system at hand, equal temperatures imply that χ and ϵ must be equal in the two phases. In Sec. III, we identified how to obtain the chemical potential and osmotic pressure from our free energy. We calculate numerically the concentration and average conformation of the protein in each of the two

coexisting phases as a function of χ, ϵ, and κ. We shall see that two qualitatively different types of phase diagrams present themselves.

We have determined the composition of coexisting phases for a variety of the model parameter values. We present these as phase diagrams, where the concentration, ϕ_{Na}, in each of the two coexisting phases is shown as a function of χ for a fixed value of ϵ and κ. By projecting the phase diagram onto the ϕ_{Na}-χ plane, we do not explicitly consider the η_{N} direction. However, to get an impression of how the fraction of proteins in the non-native state varies across the phase diagram, we present cuts at fixed values of χ.

For a given value of κ, the two different types of phase diagrams that we find are separated by a critical value of ϵ. Let us first consider the class of phase diagrams for values of ϵ below this critical value. The phase diagram for $\kappa = 1.05$ and $\epsilon = 1$ is representative of this class and is shown in Fig. 6(a). The phase diagram shows two distinct regimes where phase separation occurs: the first is indicated by a solid curve (coloured red) and the second by a dashed line (coloured blue). For both binodal curves, the coexisting phases are joined by horizontal tie lines at constant χ.

In the low-concentration regime, phase separation results in a dilute phase containing proteins in their native state and a dense phase of proteins in their non-native state. The equilibrium fraction of proteins in the non-native state for $\chi = 20$ is presented in Fig. 6(b), where the coexisting states are indicated by red filled circles. Here, phase separation occurs because it is favourable to have a dilute phase of proteins in the native state and a dense phase of proteins mostly in their non-native state, where attractive interactions compensate the free energy penalty associated with the non-native state.

In the high-concentration regime, phase separation results in a relatively dilute phase of proteins mostly in their non-native state and a denser phase where proteins are mostly in their native state. This is indicated by the blue filled circles in Fig. 6(b). At high densities, where a large fraction of the proteins are in the non-native, expanded state, the system runs out of free volume. In response to this, the proteins give up their attractive interactions in favor of an increase in translational entropy by reverting to the smaller native state. Of course, phase separation induced by self-crowding effects cannot be observed for the case where the conformers take up the same volume, and $\kappa = 1$. We note that in the high-density regime, phase separation seems to be coupled to the underlying first-order conformational phase transition discussed in Sec. III and shown in Fig. 6(b).

By labeling a phase dilute relative to another phase, we mean that ϕ_{Na} is smaller in that phase and hence that the number density of proteins is lower. However, this does not mean that volume fraction in the dilute phase is smaller than that in the coexisting dense phase. This is indeed the case for the coexisting phases at high concentration as shown in Fig. 6(b) (blue filled circles). While for the phase with proteins mostly in their non-native state we have $\phi_{\mathrm{Na}} = 0.57$ and the phase with proteins mostly in their native state $\phi_{\mathrm{Na}} = 0.63$, the corresponding protein volume fraction in the former is $\phi = 0.66$ and in the latter $\phi = 0.63$. So, in this case the more densely packed phase is actually the "dilute" phase.

Fig. 6 shows two more important characteristics of the phase behaviour of the model two-state protein dispersion. First, Fig. 6(b) shows that for values of ϕ_{Na} intermediate to the two regimes of phase separation, the non-native state is the more stable conformation. Second, the second regime of phase separation, indicated by the dashed blue binodal in Fig. 6(a), exhibits re-entrance, meaning that a continuous change in temperature and hence in χ,[32] leads to the transition from a homogeneous to a phase-separated and again to a homogeneous equilibrium state.

In Fig. 7 we show the phase diagram for $\kappa = 1.25$ and $\epsilon = 2.0$, which is representative of the second class of phase diagrams advertised above. Fig. 7(a) shows the phase diagram as a function of the protein concentration, ϕ_{Na}, which is a conserved quantity and Fig. 7(b) that as a function of the protein volume fraction, ϕ, which is a non-conserved quantity. Again there are two concentration regimes where phase separation occurs, indicated by the red and the dashed blue binodals, with coexisting phases that are joined by horizontal tie lines at constant χ. The corresponding Fig. 8 shows that for the low-concentration branch of the left phase gap the proteins are mostly in the native state, while for the high-concentration branch the proteins are mostly in the non-native state. For the

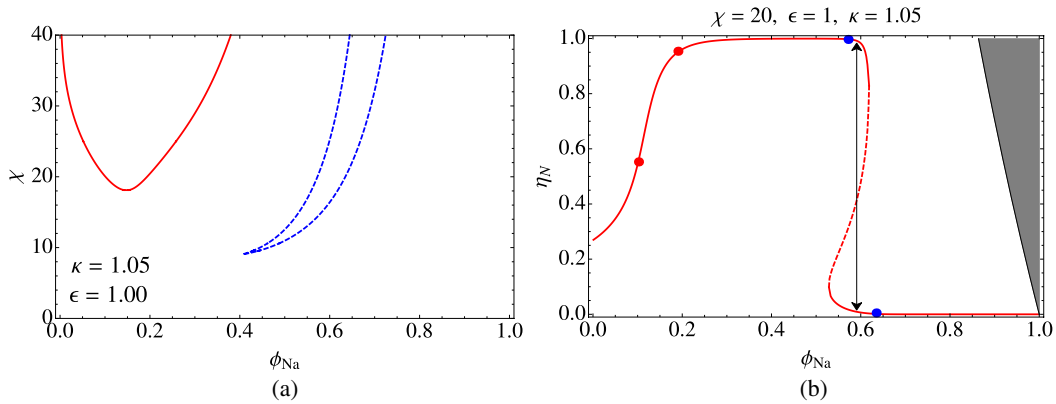

FIG. 6. (a) Phase diagram for $\kappa = 1.05$ and $\epsilon = 1$. The solid red curve gives the protein concentration of coexisting phases, joined by horizontal tie lines with constant χ. At higher protein concentrations there is a second regime where phase separation occurs, indicated by the dashed blue line giving the protein volume fractions of coexisting states which are joint by horizontal tie lines at constant χ. (b) $\eta_{\mathrm{N}}^{\mathrm{eq}}$ as a function of ϕ_{Na} for $\kappa = 1.05$, $\epsilon = 1$, and $\chi = 20$ showing the two sets of the coexisting phases as red and blue dots, respectively. The location of the first-order conformational phase transition is indicated by the double arrow.

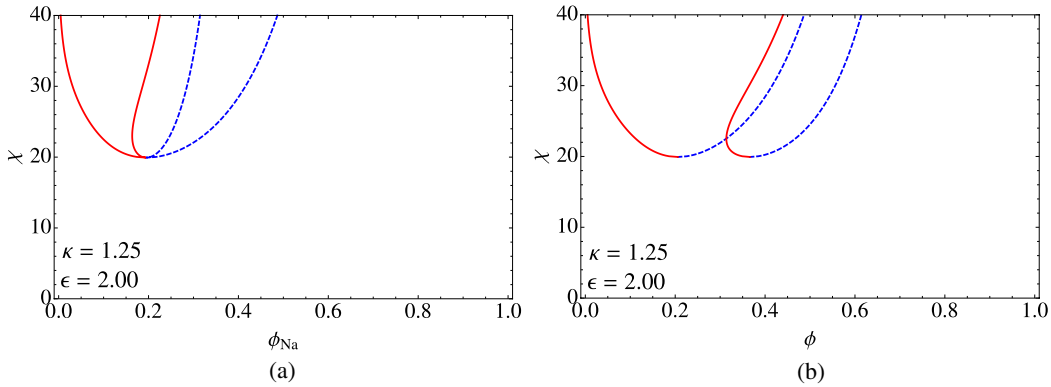

FIG. 7. (a) Phase diagram for $\kappa = 1.25$ and $\epsilon = 2.0$ showing two binodals. The solid red curve denotes the protein concentration of coexisting phases that are joined by horizontal tie lines at constant χ. At higher protein concentrations there is a second regime where phase separation occurs, with the dashed blue line indicating the protein concentration of coexisting states joined by horizontal tie lines at constant χ. (b) The same phase diagram but now shown as a function of protein volume fraction, ϕ. Coexisting states are joint by horizontal tie lines. However, because ϕ is not a conserved order parameter, the interpretation of this phase diagram is not trivial.

second, right phase gap the opposite is true, with non-native proteins in the low-concentration branch and native ones in the high-concentration branch.

As is evident from Fig. 7(a), the two regimes of phase separation at low and high densities merge at the lowest value of the interaction parameter $\chi \approx 20$. However, if expressed in terms of ϕ instead of ϕ_{Na}, it transpires that the situation is more complex because the volume fractions of the coexisting phases do not merge into something that resembles a critical point. Hence, what appears to be a critical point in Fig. 7(a) in fact is not: the fraction of proteins in the non-native state remains different upon approach of the "critical point" where the concentrations become equal. This means that the phase gaps of both first-order phase transitions do not exhibit a critical end point. The transition remains first-order because it is connected to the appearance of the first-order conformational transition described in Sec. III. This is in contrast to the first class of phase diagrams discussed above, where both phase gaps do end in an actual critical point.

At the "critical point" of Fig. 7(a), both regimes of phase separation have one of the two coexisting phases where most proteins are in the native state and one where most are in the non-native state. At this "critical point," the two regimes of phase separation can no longer be distinguished from each other. On the other hand, if expressed in the protein volume fraction, ϕ, in Fig. 7(b), the two coexisting phases are different, and the "critical point" of Fig. 7(a) splits into two points at different protein volume fractions. In each of these points, one of each of the branches of the binodal meets with one of the branches of the binodal of the other regime of phase separation.

In the second class of phase diagrams, both liquid-liquid phase separations turn out to be coupled to underlying first-order conformational phase transitions of the proteins themselves. This is how the second class of phase diagrams appears to set itself apart from the first class, where only the high-density phase separation is coupled to this underlying conformational phase transition. We illustrate this in Fig. 8, where the composition of the coexisting phases of both regimes of phase separation and the location of the underlying first-order conformational phase transitions are shown for $\chi = 23$ and $\chi = 30$.

Let us now briefly return to the phase diagram of Fig. 7(b) and focus on the region just above and in between the two "critical points." From this figure we might conclude that for these protein volume fractions both regimes of phase separation are accessible, for the protein volume fraction is intermediate to the protein volume fractions of the coexisting phases of both regimes of phase separation. However, this interpretation is incorrect: the protein volume fraction is a non-conserved

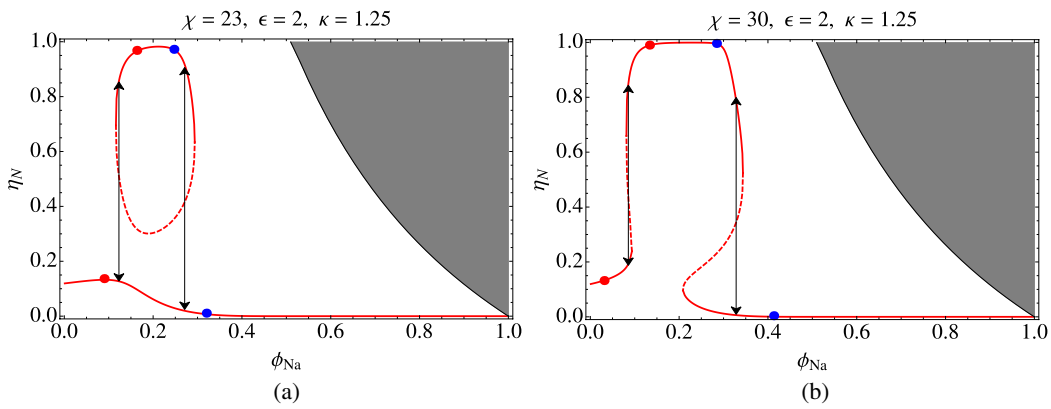

FIG. 8. (a) η_N^{eq} as a function of ϕ_{Na} for $\kappa = 1.25$, $\epsilon = 2.0$, and $\chi = 23$ showing the two sets of two coexisting states as red and blue filled circles, respectively. The first-order conformational phase transition is indicated by the double arrow. (b) The same type of graph but for $\kappa = 1.25$, $\epsilon = 2.0$, and $\chi = 30$.

quantity and the two regimes of phase separation occur at different protein concentrations that do not overlap, see Fig. 7(a). The protein concentration, ϕ_{Na}, is a conserved quantity. As a consequence, the phase diagram as shown in Fig. 7(a) must be used to determine if phase separation can occur, and if it occurs, what the composition of the two coexisting phases are and not the one shown in Fig. 7(b).

For other values of ϵ and κ, the phase diagrams are qualitatively similar to the phase diagram as shown in Figs. 6(a) or 7(a). In Sec. V we provide a summary of our findings.

V. SUMMARY AND DISCUSSION

In this paper, we present a model for protein liquid-liquid phase separation in which the protein conformation is not conserved and there is an equilibrium between an inert native conformation and an excited, expanded non-native conformation. The non-native conformation in our model is not only larger in size but also interacts attractively with proteins with the same conformation. Our calculations show that volume exclusion stabilizes the native state at high protein concentrations due to self-crowding, that is, due to a lack of free volume.

We find that there are two different first-order conformational phase transitions at fixed protein concentration. These first-order conformational phase transitions are coupled to two different regimes of liquid-liquid phase separation, where the regime at higher protein concentration is induced by self-crowding effects. Interestingly, the underlying physics of the two types of phase separation, and the associated first-order conformational phase transitions hidden behind the associated liquid-liquid phase separation, is identical.

From our theory we conclude that there are two phenomena that are caused by self-crowding, itself caused by the larger volume of the non-native conformation that reduces the free volume of the solution. These are the stabilisation of the native state and the presence of a second regime of phase separation at high protein concentrations. This is not dissimilar to what is seen in more conventional studies of macromolecular crowding where crowding agents are added to a protein solution, leading to either phase separation or aggregation depending on the specific interaction between the proteins. Here too, a decrease in free volume drives these processes.[9,22] The crucial difference of course is that in our case free volume is primarily linked with the volume the particles themselves take up, whereas in the more conventional case it is caused by the mutual excluded volume of groups of particles.

An obvious question is how the predictions of the model compare with experimental observations on liquid-liquid phase separation in protein dispersions. To our knowledge, protein dispersions only display one regime of phase separation, rather than the two predicted by our model.[33,34] A possible reason for this is that the second regime of phase separation occurs at protein concentrations where the solution gels or crystallizes.[35] Neither of these phenomena are included in our model. However, we expect similar effects to be relevant to crystallization especially since the structure of crystals is largely determined by excluded-volume effects,[30] which includes effects related to changes in the size and shape of the proteins.[36,37]

In summary, we have shown that changes in effective volume induced by switching between a native state and a non-native state for which water is a poorer solvent should strongly affect the phase behaviour of protein solutions. In effect, we find that in that case self-crowding leads to a re-stabilization of the native state at high protein concentrations.

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