Modeling equilibrium clusters in lysozyme solutions

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Abstract – We present a combined experimental and numerical study of the equilibrium cluster formation in globular-protein solutions under no-added salt conditions. We show that a cluster phase emerges as a result of a competition between a long-range screened Coulomb repulsion and a short-range attraction. A simple effective potential, in which electrostatic repulsion is fixed by experimental conditions and attraction is modeled with a generalized Lennard-Jones potential, accounts in a remarkable way for the wavevector dependence of the X-ray scattering structure factor.

Competition between short-range attraction and long range repulsion provides an efficient way to stabilize aggregates whose shape and characteristic size result from the delicate balance between these opposing forces [1–6]. Under appropriate external conditions, particles interacting with such a mixed potential may form equilibrium cluster phases. This is a state of matter in which the stable structure of the solution is characterized by the presence of equilibrium aggregates of particles, the colloid analog of micelles [7].

Cluster phases have been recently observed in colloidal systems as a result of the competition between short-range depletion attraction and long-ranged electrostatic repulsion [4,5]. Confocal microscopy has provided detailed information on the size and shape of these clusters. Theoretical and numerical studies suggest that cluster-phases can also be observed in different systems such as star-polymer solutions [8] or charged liposomes [6]. The typical signature of the equilibrium cluster phase is a pre-peak in the structure factor, signaling a preferential distance set by the competing forces on different length scales. Such a feature has been recently reported in solutions of globular proteins [4,9,10], implying a generality of the mechanism by which bulk aggregation is disfavored and finite-size clusters are formed and persist in equilibrium. This similarity suggests the possibility of an approach to globular proteins based on the assumption of an effective interaction potential, in which the competition between repulsion and attraction is built in.

In proteins, short-range attraction is normally attributed to a combination of van der Waals attraction, hydration forces, and hydrophobic interactions [11,12]. While in colloid-polymer mixtures such short-range attraction is well characterized in terms of polymer size and concentration, in protein solutions it is poorly understood. However, clear evidence for its presence is provided by studies at increasing ionic strength of the second virial coefficient [13–15] and by the determination of gas-liquid coexistence lines [16–18], which are metastable with respect to crystallization as for short-ranged attractive colloids [19]. Long-range repulsion arises from screened electrostatic interactions, associated to the net charge of the protein in pH-controlled protein solutions.

Among globular proteins, lysozyme has become the prototype for scientific investigations. Under no-added salt condition, the lysozyme structure factor shows a clear cluster pre-peak whose position is essentially independent of protein concentration and weakly dependent on temperature [4]. Hence, it is particularly important to assess under which conditions an effective potential can be designed which accounts for such typical features. Previously, theoretical studies have attempted to study lysozyme solutions for different solution parameters (*e.g.* pH, density, salt concentration), either with an effective continuum model for electrostatic interactions between proteins carrying discrete charges [20] or with explicit primitive models [15].

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At high ionic strength, modeling for the calculation of the phase diagram usually relies on purely attractive potentials [21]. Some models have incorporated the "patchyness" and/or the non-sphericity of the interactions [20,22], associated with the discrete distribution of charged and hydrophobic sites, whose relative distances could compete with the relevant distances incorporated in an effective spherical potential. But, to our knowledge, for none of these models, despite the large number of involved parameters, an equilibrium cluster phase has been predicted.

In this letter we present a combined experimental and numerical study on the equilibrium cluster formation in suspensions of a globular protein in the absence of added salt. We propose an effective simple potential for the lysozyme-lysozyme interaction in water based on a short-range attractive potential complemented by a Yukawa screened electrostatic repulsion. Parameters in the Yukawa potential are fixed by the known size and charge of the protein and the composition of the solvent. In contrast to previous studies on colloid-polymer mixtures where the contribution of background ions is dominant [23,24], counterions coming from the surface charge induce a highly concentration-dependent behavior of the amplitude and screening length of the Yukawa potential, which is properly taken into account [25]. The depth and width of the resulting potential is chosen by best-fit with the structure factor at one reference concentration, and kept fixed for all other studied state points. The derived potential is capable of accurately describing the measured static properties at low and intermediate concentrations.

The globular protein used is hen egg white lysozyme (Fluka, L7651). Its molecular weight is 14.4 kDa and its shape ellipsoidal with linear dimensions of $3 \times 3 \times 4.5$ nm [17]. For simplicity, we neglect the asymmetry in shape, and we model it as a sphere of diameter $\sigma = 3.4$ nm. Lysozyme is dispersed in a solution of D_2O (99.9%, Cambridge Isotope Laboratories) containing 20 mM Hepes buffer salt. A detailed description of the sample preparation procedure can be found elsewhere [4]. The $p\mathrm{H} = 7.8$ is adjusted with sodium hydroxide and held constant within ± 0.1 units for all volume fractions investigated. Under these conditions, the net charge of the protein is known from titration experiments to be $Z_0 = +8e$ [26]. The ionic strength of the solvent, estimated by conductimetry, is 8 mM, corresponding to a Debye screening length at infinite protein dilution of $\xi \simeq 3.4$ nm. SAXS experiments are performed with a pinhole camera (NanoSTAR from Bruker AXS) equipped with a sealed tube (Cu K_α), a thermostatically regulated sample chamber and a two-dimensional gas detector (see ref. [9] for further details). Samples are measured at volume fractions ranging from $\phi = 0.085$ to 0.201, where $\phi = \pi\rho\sigma^3/6$, with ρ the protein number density, is systematically measured by UV-visible spectroscopy.

We perform molecular dynamics simulations (MD) of a system composed of $N = 2500$ particles of diameter σ and mass m in a cubic box of size L, as a function of volume fraction ϕ and temperature T. The excluded-volume term plus the short-range (SR) attraction are modeled for simplicity with the generalized Lennard-Jones 2α-α potential [27],

$$V_{SR}(r) = 4\epsilon\left[\left(\frac{\sigma}{r}\right)^{2\alpha} - \left(\frac{\sigma}{r}\right)^{\alpha}\right], \qquad (1)$$

where α essentially controls the width of the attraction and the steepness of the hard-core, while $-\epsilon$ is the depth of the SR potential. The parameters σ and k_BT (where k_B is the Boltzmann constant) are chosen as units of length and energy, respectively. The short-range potential is complemented by a long-range repulsion, modeled with a Yukawa potential. Its amplitude and screening length are fixed by the experimental conditions and follow the generalized one-component macroion model (GOCM) [25,28],

$$V_Y(r) = k_BTL_BZ_0^2X^2\frac{e^{-r/\xi}}{r}, \qquad (2)$$

where $L_B = e^2/(4\pi\varepsilon_0\varepsilon k_BT)$ is the Bjerrum length, $\xi = [4\pi L_B(\rho|Z_0| + 2\rho_s)z^2]^{-1/2}$ is the Debye length, with ρ the protein number density, Z_0 the net charge on a protein, ρ_s the salt number density in the buffer, z the valence of the dissolved ions ($z = 1$) and X a correction factor that depends on both ϕ and ξ (eqs. (11)-(15) in [25]). The resulting Yukawa potential gives a realistic description of the effective repulsion between proteins for the relatively high-volume fractions investigated. Here the explicit ϕ-dependence of X incorporates the effect of screening of a protein by other proteins, whereas the contribution of additional counter-ions with increasing ρ enters in the calculation of ξ. Under the chosen condition of low background electrolyte, we find it important to properly describe the change of ξ and X with ϕ. Under excess salt, or infinite dilution, the GOCM reduces to the repulsive part of the classical DLVO potential. Particles are assumed to interact simultaneously via $V_{tot} = V_{SR} + V_Y$, shown in fig. 1 for various ϕ values. The integration time-step is fixed to $\Delta t = 5\ 10^{-3}$ in units of $\sqrt{m\sigma^2/\epsilon}$. A cutoff at $r_c = 8\xi$ is applied to reduce the computational effort, without significantly altering the model, since $V_{tot}(r_c) < 10^{-3}$.

Whilst the theoretical work of Belloni allows us to model the electrostatic repulsion without any fitting parameter, no theory is capable to properly account for the attractive part. However, in the case of lysozyme and numerous other globular proteins, attraction is known to be short-ranged and of moderate strength [29]. Previous scattering studies on lysozyme suspensions under high salt conditions have shown that the attractive part of the effective potential does not significantly depend on temperature [14,30,31]. Following these indications, we determine the two unknown parameters ϵ and α in the generalized Lennard-Jones potential by running simulations at fixed $\phi = 0.188$, and searching for the α-ε values best matching the experimental peak positions of $S(q)$ at 5 °C.

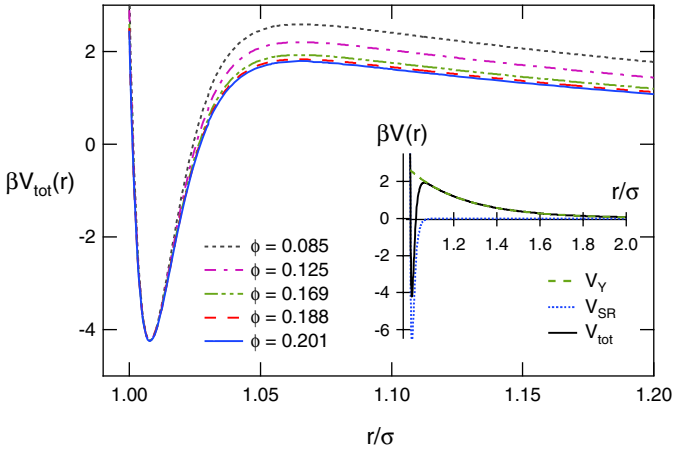

Fig. 1: Potential $V_{tot}(r)$ as a function of interparticle distance r for various ϕ. The total potential well depth is $V_{min} = -4.22 k_B T$ and its range is $\approx 3.6\%\sigma$. The inset details the construction of V_{tot} with a short-range attractive potential V_{SR} and a long-range screened Coulomb repulsion V_Y.

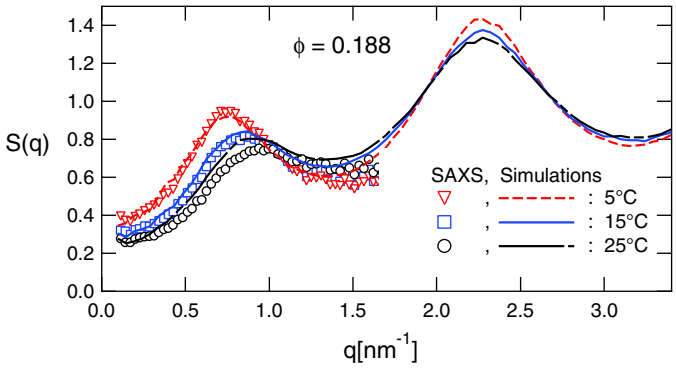

Fig. 2: Comparison of the structure factors $S(q)$ obtained by MD simulations (lines) with SAXS (symbols), for lysozyme suspensions of volume fraction 0.188 at corresponding temperatures.

The results from simulations obtained with $\alpha = 90$, corresponding to an attraction range of $3.6\%\sigma$ and $\epsilon = 6.66 k_B T$ are compared with the measured $S(q)$ in fig. 2 at $\phi = 0.188$. The position of the cluster peak and its T-dependence are properly accounted for the chosen α and ϵ values. It is important to point out that also the location of the nearest neighbor peaks at $\approx 2.25\,\mathrm{nm}^{-1}$ coincides with previous SANS measurements [4,9]. We also note that the low q-limit is rather well described, suggesting that the present potential properly accounts for the system compressibility ($\propto S(0)^{-1}$) for all temperatures. This remarkable agreement infers that the use of an effective potential captures the essential features necessary to describe the clustering process in lysozyme suspensions.

To validate the resulting potential, we turn to examine other densities. We have verified that the hypothesis of constant total depth, as opposed to the hypothesis of a constant ϵ, is necessary to properly describe $S(q)$ at all densities. We thus introduce a density dependence in ϵ

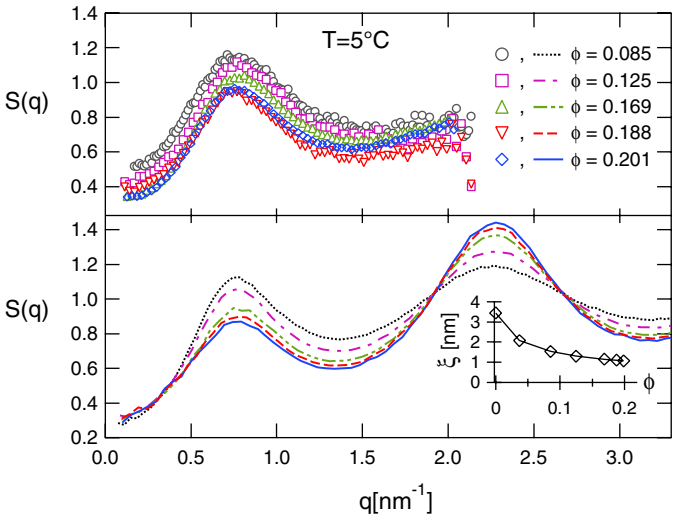

Fig. 3: ϕ-dependence of the static structure factor $S(q)$ measured by SAXS (upper panel) and obtained by MD simulation (lower panel) with potentials of fig. 1. The dependence of the Debye length ξ on volume fraction is shown as inset.

to counterbalance the density dependence of V_Y in such a way that βV_{min} remains constant at all densities, as shown in fig. 1. In this respect, the potential is built on: i) the Belloni theoretical approach for the electrostatic contribution and ii) the assumption that the depth of the resulting total potential is constant at all densities. Considering the short distances at which attraction sets in, one possible explanation of the constant V_{min} constraint arises from the spherical average requested to generate an effective centro-symmetric potential starting from a non-spherical patchy protein, mediating the location of attraction and repulsion sites [20,22].

In fig. 3 numerical results are compared with the corresponding experimental data at $T = 5\,^{\circ}\mathrm{C}$ for volume fractions ranging from $\phi = 0.085$ to 0.201. Again, the numerical $S(q)$ properly reproduces the features observed experimentally. With increasing ϕ, the cluster peak position does not significantly change while its amplitude systematically decreases. Moreover, the agreement is quantitative for the cluster and nearest-neighbor peak positions as well as for their amplitudes. Also the compressibilities are well reproduced, with an initial decrease with increasing ϕ, saturating to a roughly constant value.

The ability of the potential to correctly reproduce the structure of the system suggests to analyze the equilibrium configurations provided by MD. Particles are considered to be in the same cluster when the separation between pairs of nearest-neighbor particles is smaller than $r_{max} = 1.065\sigma$, a value corresponding to the distance at the maximum in V_{tot} (see fig. 1). Figure 4a) shows the resulting distribution $n(s)$ of clusters of size s, for several state points. We note that these results are qualitatively unaffected by different choices of the bonding distance. At the smallest ϕ, the suspension is essentially composed of small clusters with a distribution that rapidly and

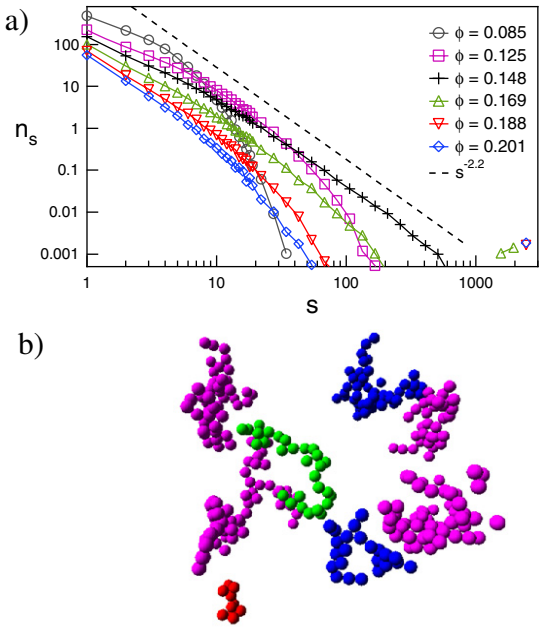

Fig. 4: a) Cluster size distribution $n(s)$ for various ϕ at $T = 5\,^{\circ}\mathrm{C}$. The distribution for small sizes follows random percolation dependence of $s^{-2.2}$. For $\phi > 0.148$ large clusters are found indicating that the system is percolating. b) Snapshot at $\phi = 0.125$ with few selected clusters of size $n = 10, 30, 82, 203$.

monotonically decays to zero. As ϕ increases, larger clusters form and the distribution progressively develops a power law behavior $n(s) \propto s^{\tau}$, with an exponent $\tau \approx -2.2$ consistent with random percolation.

We can try to exploit the micelle analogy in order to understand the variation of the cluster size distribution shown in fig. 4. In the case of charged micelles at low ionic strength, one expects a dramatic change in the micellar growth scenario at a crossover concentration where the screening length becomes comparable to the micellar length [32]. At this point, a sharp transition to accelerated micellar growth with a very broad size distribution occurs. In the studied concentration range, we indeed observe an enhanced cluster growth and a large broadening of the size distribution close to $\phi = 0.085$, where the ration ξ/σ has decreased to a value of 0.5. From that point, we indeed observe an enhanced cluster growth and a large broadening of the size distribution. This is due to the effect of decreasing electrostatic repulsion on top of that of the increase in number density, resulting in a faster growth with respect to standard short-ranged attractive systems.

For $\phi > 0.148$ percolating states are found where the system forms a space-spanning cluster. From the analysis of the MD trajectories one observes that clusters are highly transient. Even above the percolation threshold, the system is still in a fluid phase, and percolation does not imply gelation, for which a long-living network is necessary. Interestingly, the observed transient percolating states resemble those of the transient networks found in the semidilute regime of charged micelles, where under the same salt-free conditions a persisting low-q peak is also present [33]. The observation of isolated clusters, as depicted in fig. 4b), reveals that the clusters are highly random. We find both space-filling spherical shapes as well as filamentous structures, made primarily of single chains, that are very different from those observed in other more repulsive systems [5,24]. Moreover, in the investigated region of phase space, there is not a preferred cluster size, since a peak in $n(s)$ is never observed.

Clusters observed under the present no-salt conditions are generated by the competition between attraction and repulsion, as clearly indicated by the presence of the pre-peak in the structure factor and should not be considered as the transient clusters that are commonly observed on approach to a phase-separation boundary in short-range potentials, whose spectral signature is a peak in $S(q)$ at $q \to 0$. In the investigated T-range, attraction is always sufficiently counter-balanced by the electrostatic repulsion, allowing for the existence of stable clusters and preventing phase separation. The resulting clusters are highly polydisperse and their structural development with volume fraction leads to transient networking, with hallmarks of random percolation.

In summary, we demonstrated that equilibrium cluster formation in complex protein solutions can be reproduced using a unifying "colloid-approach", with a simple one-component effective potential between proteins composed of a short-range attraction and a longer-range repulsion. This indicates the existence of a fundamental principle for self-assembly in biological solutions. It is interesting to note that aggregation and bundle formation in biological polyelectrolytes such as DNA [34], microtubules or f-actin [35,36] has also been looked at as an equilibrium process based on a balance of opposing forces. While the attraction is of rather different electrostatic origin and thought to arise from the presence of multivalent counterions, it also underlines the importance of self-assembly due to a combination of non-specific short-range attractive and long-range repulsive forces in these systems.

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