What is the physical origin of nanoclusters?

Theory of condensate size control by molecular charge asymmetry
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ACS Macro Letters **14**(10), 1484-1491 (2025)

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The concept of liquid-liquid phase separation has provided us with a convenient language to describe how cells organize components beyond membrane-bound organelles. However, the more carefully we look, the less adequate the "simple" classical phase-separation picture appears for describing biological condensates. One striking example is the observation of nanoclusters—clusters of macromolecules a few hundred nanometers in size. A growing body of work reports the existence of such nanoclusters both *in vitro* and *in vivo* below the threshold concentration for macroscopic phase separation [1–6]. The physical origin of these nanoclusters, however, remains debated.

Here, we comment on the recent paper by Luo and co-authors where they combined continuum field theory with coarse-grained molecular dynamics simulations to study the phase behavior of charged mixtures. In particular, they show how electrostatic repulsion generated by molecular charge asymmetry can thermodynamically stabilize finite-sized clusters. Their work provides valuable insights into what electrostatics can and cannot do for charged mixtures at equilibrium. Below, we outline the key findings of Luo *et al.* and discuss how far electrostatics can take us toward understanding nanoclusters in real biomolecular systems.

Experimental observations

Recent work points to nanoclusters as robust features of both reconstituted and cellular systems [1–6]. Because the cellular environment is highly heterogeneous and continuously driven, it is difficult to identify the physical origin of nanoclusters *in vivo*. Therefore, we focus primarily on simple *in vitro* systems with one or two macromolecular components.

For FET-family RNA-binding proteins such as FUS, light scattering and single-molecule measurements reveal heavy-tailed cluster-size distributions at concentrations as low as one-tenth of the threshold concentration, where classical nucleation theory would predict essentially only monomers [1]. Kinetically, nanoclusters formed below the threshold concentration initially grow with time but then cease to coarsen at a finite size on the order of hundreds of nanometers [3]. These nanoclusters nonetheless readily exchange components with the surrounding solution. As the bulk concentration increases toward the apparent threshold concentration for macroscopic phase separation, the population of clusters grows and the

size distribution shifts smoothly to larger clusters, before micron-sized droplets appear [1, 3]. Interestingly, replacing negatively charged residues (ten aspartate and four glutamate) with glycine in the RNA-binding domain of FUS enhances nanocluster formation but impairs macroscopic phase separation, underscoring the importance of electrostatics for nanocluster behavior [1].

Mechanisms of formation

The experimental observations have prompted a number of theoretical proposals, which can be loosely grouped according to whether nanoclusters are viewed as thermodynamically stable structures or kinetically trapped states.

One possibility is that nanoclusters are true equilibrium structures. (1) Multivalent proteins with sticker-spacer architectures are known to exhibit phase separation coupled to percolation, i.e., the dense phase is a percolated network. It has been proposed that such sticker-spacer polymers can also form extensive, but finite, networks below the macroscopic phase-separation threshold, and that nanoclusters correspond to the heavy tail of the dilute-phase ensemble of associative networks [1]. (2) In core-shell theories, it has been proposed that proteins can take multiple conformational states with different solvation properties, and that nanoclusters are stablized when protein configurations in the shell minimize the interfacial energy cost. In this view, nanoclusters are mesoscale analogues of micelles [7].

An alternative view is that nanoclusters are kinetically trapped, metastable states. In this picture, nanoclusters fail to grow into micron-sized droplets because adding dilute-phase monomers to nanoclusters and merging between nanoclusters face substantial free-energy barriers, possibly due to electrostatic repulsion or slow conformational rearrangements at the cluster interface [2, 4]. Therefore, on experimental timescales they appear "stable", even though the true thermodynamic ground state would be a macroscopic condensate.

Phase behavior of charged polymer mixtures

The beauty of the work by Luo *et al.* is that they deliberately chose a very minimal model. Two oppositely charged polymers P^+ and P^- interact via short-range attractions that favor phase separation, while mobile ions e^+ and e^- ensure overall charge neutrality and screening. The system is governed by a continuum free-energy functional:

$$F = \int \left[\frac{k_{\mathrm{B}}T}{v} \left(\sum_i \frac{\phi_i}{l_i} \ln \phi_i + \chi\, \phi_{P^+} \phi_{P^-} + \frac{1}{2} \sum_i \kappa_i |\nabla \phi_i|^2 \right) - \frac{\varepsilon}{8\pi} |\nabla \psi|^2 + \frac{e\psi}{v} \sum_i z_i \phi_i \right] \mathrm{d}V.$$

The first two terms inside the brackets form a Flory-Huggins free energy: $\phi_i(\mathbf{r})$ is the local volume fraction of species i (including P^+, P^-, e^+, e^-, and solvent), $k_{\mathrm{B}}T$ is the thermal energy, v is a reference molecular volume, and l_i is the degree of polymerization. The parameter $\chi < 0$ denotes a short-range attraction between P^+ and P^-. The third term, proportional to κ_i, is a standard square-gradient (Cahn–Hilliard) contribution that penalizes sharp spatial variations of composition and sets the interface width. The last two terms are the electrostatic free energy, where $\psi(\mathbf{r})$ is the electrostatic potential, ε is the dielectric constant, and z_i is the charge number. Variation of F with respect to ψ ($\delta F/\delta\psi = 0$) yields

Poisson's equation, which couples the composition fields $\{\phi_i\}$ to the electrostatic potential. The system achieves an equilibrium state when the total free energy is minimized.

The key control parameter is the charge asymmetry of the polymers, i.e., the total charge of P^+ is not exactly balanced by that of P^-. Luo *et al.* show that, for sufficiently large charge asymmetry, the competition between the interfacial free energy $F_{\rm int}$ (which favors coarsening and larger droplets) and the electrostatic free energy $F_{\rm el}$ (which penalizes net charge separation over long distances) can stabilize finite-sized clusters at equilibrium. Specifically, when

$$F_{\rm int} \equiv \int \frac{k_{\rm B}T}{2v} \sum_i \kappa_i |\nabla\phi_i|^2 {\rm d}V = F_{\rm el} \equiv \int \frac{\varepsilon}{8\pi} |\nabla\psi|^2 {\rm d}V,$$

many clusters of similar size coexist rather than merging into a single macroscopic droplet, which is supported by their coarse-grained molecular dynamics simulations.

The more delicate issue is what cluster sizes are actually selected once model parameters are constrained by physiological considerations. Here Luo *et al.* are explicit. When polymer charges, salt concentrations, and interaction strengths are chosen to be consistent with physiological values, the characteristic cluster size is only about an order of magnitude larger than the Bjerrum length $l_{\rm B}$, with $l_{\rm B} \sim 1\,{\rm nm}$. This implies cluster sizes of only a few nanometers, so that each cluster contains at most tens of molecules. Consistent with this interpretation, their simulations suggest that charge-asymmetry-driven size control operates primarily at molecular scales.

The role of electrostatics for nanoclusters

Luo *et al.* describe a mechanism of droplet size control in which ions are partially expelled, resulting in a net-charged dense phase. Because the corresponding electrostatic free energy diverges for macroscopic phase separation, microphase-separated finite-sized clusters emerge as a compromise. Similar Coulomb-frustrated phase separation has been proposed for weakly charged polyelectrolyte solutions at low salt concentrations [8]. Overall, the results suggest that electrostatics by themselves are unlikely to generate thermodynamically stable nanoclusters in the hundred-nanometer range for biomolecules under physiological conditions.

Electrostatic interactions are, nevertheless, poised to play an important role. One possibility is that electrostatics act in concert with other equilibrium mechanisms, such as core-shell architectures or associative polymer networks, that already favor finite-sized assemblies and together frustrate macroscopic phase separation. In such scenarios, nanoclusters would be genuine equilibrium structures, with their size distribution shaped by the combined effects of electrostatic interactions, multivalent binding, and mesoscale interfacial costs. Electrostatics may also be crucial if nanoclusters are kinetically trapped. In this case, electrostatic repulsion between charged nanoclusters can suppress coalescence, effectively arresting the system in a cluster-rich state over experimental timescales. Here, electrostatic repulsion does not by itself select a particular size distribution, but it provides a natural mechanism to slow down growth once clusters have formed by other means.

Discriminating thermodynamically stable nanoclusters from kinetically trapped ones will require systematic perturbations of charge numbers and patterning, long-time measurements of coarsening dynamics and cluster lifetimes, controlled cycles in external parameters (such

as salt, pH, or temperature) to test reversibility, and direct observations of cluster fusion and dissolution. In parallel, the central challenge for theorists is to understand how electrostatics, multivalent interactions, and molecular conformations collectively give rise to the robust nanoclusters seen in experiments.

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