

# A multi-step nucleation process determines the kinetics of prion-like domain phase separation

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Liquid-liquid phase separation (LLPS) has emerged as a unifying theme in biology as a mechanism for organizing biomolecules in space and time. Under biologically relevant conditions, LLPS likely proceeds through nucleation and growth, and the nucleation barrier determines the rate at which phase-separated compartments can form. To characterize the nature of the energetic barrier to nucleation, we have combined equilibrium techniques that define the phase boundaries of a prototypical prion-like domain and the size distribution of clusters below the nucleation barrier with rapid mixing time-resolved small-angle x-ray scattering (SAXS). SAXS is sensitive to structures on the nanoscale, and rapid mixing microfluidics allows us to probe the size evolution of the system as it progresses toward two-phase equilibrium. We demonstrate that homogeneous classical nucleation theory can describe phase separation of prion-like domains on length scales greater than small oligomers. However, this theory breaks down for small oligomers where an additional energy barrier to assembly exists. These results suggest that a conformational change is required for nucleation and present an experimental methodology by which nucleation can be rigorously studied.