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Interplay between Kinetics and Dynamics of Liquid–Liquid Phase Separation in a Protein Solution Revealed by Coherent X-ray Spectroscopy

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the early stage of SD, the kinetics relaxation is up to 40 times slower than the dynamics and thus can be decoupled. The microscopic dynamics can be well described by hyper-diffusive ballistic motions with a relaxation time exponentially growing with time in the early stage followed by a power-law increase with fluctuations. These experimental results are further supported by simulations based on the Cahn-Hilliard equation. The established framework is applicable to other



condensed matter and biological systems undergoing phase transitions and may also inspire further theoretical work.

iquid-liquid phase separation (LLPS) is a fundamental process that has significant consequences in many biological studies, e.g. investigations of protein crystallization, biomaterials, and diseases,² as well as for food³ or the pharmaceutical industry.⁴ In cells, LLPS can play a crucial role in driving functional compartmentalization without the need for a membrane and as a ubiquitous cellular organization principle implicated in many biological processes ranging from gene expression to cell division.5-7 LLPS is a complex process involving domain evolution of the new phases, microscopic dynamics of density fluctuation, and the thermal fluctuation of domain interfaces, as well as global diffusive motion. While both the kinetics of domain evolution and the microscopic dynamics are important for the formation and properties of the various condensates, research so far mainly focused on the domain growth kinetics and the dynamics remains largely unknown.

Dynamics of a system in equilibrium can be described using the fluctuation of the static structure factor, S(q), where the average density is constant and $\rho(t)$ fluctuates around this mean value. In this case, $\overline{S(q)}$ is constant and dynamics can be directly extracted. For a system undergoing phase separation, the interplay between dynamics and kinetics may lead to a more complex scenario, i.e. the temporal average density (and accordingly, $\overline{S(q)}$) changes and fluctuates at the same time. At the late stage of LLPS via SD, theoretical studies have shown that the domain coarsening follows dynamic scaling, i.e., during domain evolution, the domains remain essentially statistically

self-similar at all times.⁸ As a result, $\overline{S(q)}$ does not change if the measurements are performed at length scales corresponding to the average domain size.^{9,10} Thus, in principal, the timedependent fluctuations of S(q) can be described by the twotime intensity covariance, which equals the square of the twotime structure factor under the "Gaussian decoupling approximation".^{9,10} Therefore, domain coarsening dynamics can be accessed by calculating the speckle-intensity covariance.^{9,10}

However, the early stage of LLPS is much more complex because the time-dependent S(q, t) cannot be described by dynamic scaling. The interplay between kinetics and dynamics makes a quantitative description of the microscopic dynamics challenging. So far, despite the substantial process in kinetic growth studies,^{11,12} such a theory to predict or an experimental method to probe the dynamics in the early stage of SD is missing. Nevertheless, the dynamic property of LLPS is a vital input to predict the dynamic properties of the resulting condensates in biological systems. Studies of LLPS in cells have shown that the exact biological function of the resulting condensates strongly depends on their dynamic properties,

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such as a dense liquid, a gel, or a glassy state.⁵ Therefore, detection in the early stage of the condensates are needed, which requires a characterization of the dynamics of LLPS. Furthermore, as LLPS in cells is generally triggered by a gradient of constitutes instead of a temperature quench, the dynamic properties of LLPS also reflect the material transport in the crowded environment of a multicomponent system.

Despite the recent progress in the field of phase separation,^{5,13} the interplay between the kinetics and dynamics at the early stage remains elusive. What is the influence of the change of S(q) with time on dynamics? Whether one can distinguish the kinetic and dynamic contribution experimentally? What are the main features of dynamics in the early stage of LLPS? In principle, both domain growth kinetics and microscopic dynamics of fluctuations of the interface of the domains of LLPS can be simultaneously accessed experimentally using X-ray photon correlation spectroscopy (XPCS). The XPCS investigation of the fluctuations during the late, domain coarsening, stage was demonstrated vividly by the work of Malik et al. in a sodium borosilicate glass. Microscopic dynamics of systems undergoing phase transition displays rich nonequilibrium behavior on length-scales ranging from micrometer to single-protein size and on time scales from hundreds of seconds down to microseconds.¹⁵ XPCS has been used in different soft condensed matter systems.¹⁶⁻¹⁹ However, due to experimental difficulties in working with beam-sensitive samples,^{20–22} XPCS of protein-based systems became possible only recently with a special experimental procedure design.²³⁻²⁶

In this letter, we focus on the dynamics during the early stage of the spinodal decomposition. The observed dynamics come from the large-scale density fluctuation and the movement of the interface of the domains.¹⁰ Consequently, the dynamics from the equilibrium mixed or demixed bulk phases do not contribute much due to the low contrast and signal-to-noise ratio.²⁷ We apply state-of-the-art XPCS in the ultrasmall angle scattering (USAXS) geometry to investigate the domain evolution and microscopic dynamics during LLPS in aqueous protein solutions. The system consists of the globular protein bovine serum albumin (BSA) in the presence of trivalent salt yttrium chloride (YCl₃).^{28,29} The phase behavior of this system has been established to exhibit a lower critical solution temperature (LCST) phase behavior.²⁸⁻³⁰ The phase diagram of the LLPS binodal has been established,²⁸ and the kinetics of LLPS of this system has been well studied using USAXS.^{28,31} Here we aim to explore both the growth kinetics and the microscopic dynamics in order to distinguish their roles in the early stage of LLPS and the domain coarsening using XPCS in the USAXS geometry. Combined with 2D Cahn-Hilliard simulations, we demonstrate that the dynamic information can be distinguished from the growth kinetics with a factor of ~ 40 difference in time scales. The method established in this work can be used to access the dynamics during phase transitions in a broad range of soft matter and biological systems.

BSA and YCl₃ were purchased from Sigma-Aldrich. Sample preparation followed our previous work.^{28,31} The stock solutions of protein and salt were mixed at 21 °C with an initial protein concentration of 175 mg mL⁻¹ and salt concentration of 42 mM. The obtained solution was equilibrated and centrifuged, and the dense phase was used for further experiments.

XPCS experiments were performed in USAXS mode at PETRA III beamline P10 (DESY, Hamburg, Germany) at an incident X-ray energy of 8.54 keV ($\lambda = 1.452$ Å) and beam size of 100 μ m \times 100 μ m. The sample-to-detector distance was 21.2 m, which corresponds to a q range of 3.2×10^{-3} to $3.35 \times$ 10^{-2} nm⁻¹, where $q = 4\pi/\lambda \sin \theta$ and 2θ is the scattering angle. The data were collected by an EIGER X 4 M detector with 75 $\mu m \times 75 \mu m$ pixel size. The solution was filled into capillaries of 1.5 mm in diameter and first equilibrated at 10 °C for 10 min. Then, it was heated with a Linkam stage to 40 °C with a rate of 150 °C/min. The system was followed from the beginning of the temperature change. The measurement was performed twice, once with high temporal resolution and a total measurement time of 60s, and a second time with lower temporal resolution but larger total measurement time, for a total of 312 s (SI Table 1). After each measurement, the solution was cooled back to 10 °C. This condition corresponds to a spinodal decomposition based on the kinetic studies.²⁸ Details can be found in the Supporting Information (SI).

The time-resolved 2D speckle patterns collected in XPCS measurements are analyzed using a two-time correlation function (TTC) $G(q, t_1, t_1)$:³²

$$G(q, t_1, t_2) = \frac{\overline{I(t_1)I(t_2)} - \overline{I(t_1)}\overline{I(t_2)}}{[\overline{I^2(t_1)} - \overline{I(t_1)}^2]^{1/2}[\overline{I^2(t_2)} - \overline{I(t_2)}^2]^{1/2}}$$
(1)

where the average is over pixels with the same momentum transfer $q \pm \Delta q$ (calculated for q from 3.5 to 11.2 μm^{-1}). Here t_1 and t_2 are the times at which the intensity correlation is calculated. Typical results of TTC are presented in Figure 1. In the TTC of the system in the first 60 s after the start of the temperature change, a relaxation signal appears a few seconds



Figure 1. Two relaxation modes revealed by XPCS. (a) TTC for the first 60 s and (b) 312 s after a temperature jump from 10 to 40 °C for 312 s experiment at $q = 4.4 \ \mu m^{-1}$. The color bar corresponds to the $G(q, t_1, t_2)$ values. The slow mode describes the sudden decay in correlation in part b around 20–30 s, exhibiting a square feature in TTC. The Blue long-dash line shows the direction of the increase of experimental time t_{age} , and the dotted line displays the direction of the g₂ cuts. (c inset) Values g_2 obtained from part b. (c) Relaxation time τ evolution as a function of t_{age} for fast (black squares) and slow (red circles) modes at $q = 4.4 \ \mu m^{-1}$. τ of the fast mode follows an exponential growth (black solid line) before 200 s, whereas τ for the slow mode grows linearly with time (red solid line).

after the temperature jump, initially with a fast decay rate, but quickly broadening with t_{age} . The TTC of the first 312 s shows that the main relaxation along the diagonal turns into a steady state after the quick broadening. A new slow relaxation mode gradually appears (Figure 1a), and its correlation shows a decay, leading to a square-like feature in the TTC. This feature is most likely caused due to the formation of the domains.

The correlation function, $g_2(q, t_{age})$, can be determined by both horizontal and diagonal cuts from the TTC.³³ Here we focus on the diagonal ones to resolve all interesting features of TTC including the square. For comparison, the results of horizontal cuts are shown in Figure S6. $g_2(q, t_{age})$ functions can be fitted using the Kohlrausch–Williams–Watts (KWW) relation to determine the characteristic relaxation time (τ) and the shape parameter $(\gamma)^{34}$ as functions of q and t_{age} . As we can see in the inset of Figure 1c, the $g_2(q, t_{age}, t)$ functions display two-decay relaxations—a fast and a slow mode, which can be modeled using two exponential decay functions:

$$g_2 = \beta_1 \exp\left(-2\left(\frac{\overline{t}}{\tau_1}\right)^{\gamma_1}\right) + \beta_2 \exp\left(-2\left(\frac{\overline{t}}{\tau_2}\right)^{\gamma_2}\right)$$
(2)

where the index 1 corresponds to the fast component and the index 2 to the slow component of the relaxation. The parameters τ and γ are functions of both q and t_{age} . Here $\overline{t} = t_2 - t_1$ is a delay time, t_{age} is the time that passed from the start of the heating, $\beta(q)$ is the speckle contrast, which can vary from 0 (incoherent scattering) to 1 (fully coherent scattering) for ergodic processes. The g_2 functions were averaged over a small range of time t_{age} to increase the signal-to-noise ratio (SI Table S1).

Relaxation times, τ_1 and τ_2 , extracted from the KWW fits are shown in Figure 1c. The fast mode exhibits two distinct stages of the evolution of relaxation time: initially ($t_{age} < 150$ s), the value τ_1 increases exponentially, followed by fluctuations around $\tau_1 \sim 100$ s in the later stage of the experiment (Figure 1c). Similar two-stage behavior has been reported in XPCS investigations of Wigner glasses,³⁵ glassy ferrofluids,³⁶ and eggwhite²³ and has been suggested as a general feature near the glass transition as well as gelation. The results of the horizontal cuts are consistent (Figure S6).

The slow mode corresponds to the broad square-like feature observed on the TTC in Figure 1b. The relaxation time of the slow mode linearly increases as a function of t_{age} . The shape parameter γ_2 of the slow mode shows a jump in the early stage from values less than 2 to higher ones and then fluctuates at values around 2.7 (Figure S2a). Such high values indicate that there are different phenomena taking place in the early and later stages, and these stages hardly correlate with each other. Based on a comparison (Figure S3) of the behavior of the $\overline{S(q)}$ for the experiment and simulation (discussed later) and matching with the real-space picture (obtained from simulation, Figure S4), it seems that the appearance of the slow mode correlates with the transition from the density fluctuation to the coarsening.

In the early stage of LLPS, due to the strong coupling between domain growth and interface fluctuation, the resulting relaxation rates (Figure 1c) may have both contributions. Dynamical scaling cannot describe the time-dependent scattering profiles in the early stage as expected (Figure S1). In order to distinguish or decouple the contributions, we determine and compare the kinetic relaxation rate with the XPCS results.

The evolution of a kinetic rate Γ_{kinetic} at a specific q can be calculated through the changes in the scattering intensity $I(q, t_{\text{age}})$:³⁷

$$\Gamma_{\text{kinetic}}(q, t_{\text{age}}) = \frac{1}{2I(q, t_{\text{age}})} \frac{\mathrm{d}I(q, t_{\text{age}})}{\mathrm{d}t_{\text{age}}}$$
(3)

The scattering intensity $I(q, t_{age})$ was calculated for each value of t_{age} based on USAXS experiments as the mean intensity $I(q \pm \Delta q, t_{age})$ of all pixels inside the ring $q \pm \Delta q$ (Figure 2a), but



Figure 2. Decoupling between kinetics and dynamics. (a) Representative structure factor evolution with t_{age} at $q = 4.4 \ \mu m^{-1}$. (b) Comparison of relaxation rates for the fast mode of XPCS (orange) and for the slow mode of XPCS (coral red) with kinetic relaxation rate (purple, obtained from USAXS) for $q = 4.4 \ \mu m^{-1}$. Yellow and green regions in parts a and b correspond to the density fluctuation and domain coarsening stages of LLPS, respectively.

only the absolute values of dI/dt_{age} are used for comparison. For the investigated *q*-region the temporal evolution of the scattering intensity is caused by the spinodal peak position shifting from high to low *q*-values (Figure S3a).^{28,31}

Corresponding typical temporal evolution of S(q) is shown in Figure 2a. The kinetic decay rate calculated using eq 3 shown in Figure 2b exhibits a quick decrease with aging time initially and becomes nearly a constant after about 100 s. For comparison, the decay rates of both slow and fast modes from XPCS measurements are also shown in Figure 2. It is interesting to see that the slow-mode decay rate is similar to the kinetics during the early stage of coarsening.

The fast mode starts at $t_{age} \sim 7$ s, before which the dynamics is too fast to be detected with the current XPCS measurements (limited by values of exposure and delay times). The decay rate is up to 40 times higher than the kinetic relaxation in the early stage and about 1 order of magnitude faster in most of the coarsening stage (up to ~150 s). Thus, these two effects are decoupled, and the fast mode corresponds to the microscopic dynamics of the movement of the interface of the domains and large-scale density fluctuations. The results are similar for other *q*-values in the [3.5, 11.2] μm^{-1} range.

Now we can interpret the fast mode as a reflection of the principal dynamics of the system during LLPS. The *q*-dependency of its relaxation time is shown in Figure 3a. In the early stage, $t_{age} < 20$ s, τ follows an inverse linear relationship with q, $\tau \sim q^{-1}$. With increasing time, it becomes nonlinear with $\tau \sim$ between q^{-1} and q^{-2} . In the mean time, the shape parameter $\gamma > 1$ (Figure 3b). These results demonstrate



Figure 3. Hyper-diffusive dynamics revealed by XPCS: (a) q-dependent on τ and (b) shape parameter γ as a function of t_{age} for the fast mode dynamics of XPCS. Gray lines represent fits with $\tau \sim q^{-1}$ (dashed) and $\sim q^{-2}$ (dotted).

the domain fluctuation dynamics to follow the hyper-diffusive ballistic motion in the early stage. The hyper-diffusive dynamics seems to be a rather universal behavior for systems in the nonequilibrium state 23,35,36,38 .

As discussed in the Introduction, during the domain coarsening step, due to the dynamic scaling effect, the covariance of the TTC is directly connected with the fluctuation of S(q), i.e. the dynamics of domain fluctuation. Here we perform simulations to verify if the kinetic relaxation rate can be decoupled with the dynamics in this stage as well. A system undergoing spinodal decomposition can be described by the Cahn-Hilliard equation (CH),^{39,40} which gives the concentration at each point of the simulated map with time. After rescaling of parameters⁴¹ and adding a temperature jump,⁴² we can write

$$\frac{\partial u(\mathbf{r}, t)}{\partial t} = \nabla \left[m(u) \nabla \left(-\frac{T_{\rm c} - T}{T_{\rm c}} u + u^3 - \nabla^2 u \right) \right]$$
(4)

Where, $u(\mathbf{r}, t)$ is rescaled time-dependent local concentration, m(u) is the mobility function, and T_c is the critical temperature. Further details of the simulation can be found in the SI. This model is coarse-grained and intentionally simplified. The goal of the simulation is to generalize the results from the specific sample. Full large-scale simulations^{12,43} are beyond the scope of this study.

Based on the simulations, the evolution of the 2D field of concentration was calculated (Figure S4c-f) and the corresponding scattered speckle pattern $I(q, t_{age})$ was calculated (i.e., image in reciprocal space) for each time step as a square of the magnitude of the 2D fast Fourier transform of the fluctuations of the concentration.⁴⁴ The dynamics of the simulated LLPS process was further characterized using eq 2. The results were averaged over three simulations with different initial conditions.

The main results of the simulations are presented in Figure 4. They are highly dependent on the *q*-value. In order to qualitatively compare the simulation with experimental data, we focus on the similar *q*-region in comparison to the *q* value of the early stage peak position of scattered intensity (Figure S3). The total time of the simulation was estimated from the behavior of the scattering intensity for the chosen *q* with time t_{age} . For the described parameters, the simulated TTC reproduces the main features of the experimental TTC (compare Figure 4a and Figure 1b). TTCs from the simulation also show a square feature as observed in the experimental TTC (Figure S7).



Figure 4. Kinetics and dynamics of LLPS simulated using CH equation: (a) Representative TTC for $T = 0.05T_c$ with a total simulation time of $t_{age} = 3105$. (b) Relaxation times of the two modes as a function of t_{age} . (c) Comparison of decay rates for the slow (coral red) and fast (orange) modes of dynamics with kinetics (purple) for simulated LLPS. Here $q_{simulation} = 22$ pixels which is similar to the experimental $q = 4.4 \ \mu m^{-1}$ (see Figure S3a and c).

The g_2 functions from simulations also contain two relaxations, which demonstrates the existence of the two modes of dynamics in a general case. The relaxation time for the slow mode linearly increases with time (Figure 4b). For the fast mode, it increases with a modulation before reaching a steady state. The shape parameters $\gamma > 1$ show behavior similar to the experimental results (Figure S2b). Figure 4c compares the relaxation rates. The relaxation rate of the slow mode obtained from correlation analysis exhibits behavior similar to the kinetics. Importantly, a simple classical model used for the current simulation of the phase transition demonstrates that the dynamics is much faster than the kinetics. This generalizes the result of time scale separation and widens its application from the specific one to a broad range of LLPS systems.

It is worth noting that the simulation does not consider thermal motion, the limited heating rate, and the viscoelastic properties of the real system. These may be the reasons that the early stage of simulation results does not show the exponential growth of τ (compare Figure S5 with Figure 1a). The early stage of LLPS is sensitive to the thermal motion.⁴⁵ It may result in the loss of the spatial correlations, which influences the visibility of the kinetic relaxation rate (being much slower than thermal motion effects) on the TTC. Thus, the dynamics is dominating in the experimental TTC. In the simulated TTC this effect is not included, and the kinetic relaxation rate can be seen in the early stage. The influence of the thermal motion decreases with the increase of the domain sizes, resulting in the appearance of the kinetic relaxation rate in the TTC (square feature) and finally becomes negligible in the coarsening stage. Another possible phenomena influencing the spatial correlation is the Marangoni effect.⁴³

In summary, we have studied the nonequilibrium dynamics and kinetics of a protein solution undergoing a liquid-liquid phase separation using USAXS-XPCS. The results show that microscopic dynamics is up to 40 times in the early stage and still at least one magnitude faster in the late coarsening stage than the kinetic relaxation rate. Thus, these two components could be decoupled. In the early stage, the microscopic dynamics is not Brownian dynamics of the proteins, which would have required relaxation time $\propto q^{-2}$ and shape parameter $\gamma \sim 1$. Instead, this dynamics can be well-described using a hyper-diffusive ballistic motion, i.e. the relaxation time $\propto q^{-1}$ and shape parameter $\gamma \sim 2$. Furthermore, the relaxation time of the dynamics exponentially increases with time in this early stage. In the late stage, where the domain coarsening is the main process, the relaxation time increase with time following a power law.

Cahn–Hilliard simulations support the experimental results and broaden the conclusions to LLPS phenomena in general, making our results applicable for other soft matter and biological systems undergoing phase transition, where kinetics and dynamics are intertwined. For example, LLPS in living cells leads to various types of condensates with distinct dynamic properties, such as dense liquid, gel, and glass-like states.⁵ These distinct dynamic properties influence not only the kinetics of LLPS but also the material transport and their biological functions in the crowded cellular environment. Finally, we emphasize that this work can be extended to different length and time scales using XPCS in SAXS mode and the fast development of X-ray free-electron laser (XFEL) facilities, so the dynamic behavior ranging from single protein to the domain coarsening could be covered.

ASSOCIATED CONTENT

G Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.jpclett.1c01940.

Information about experimental details, simulation, and horizontal cuts analysis of TTC (PDF)

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Notes

The authors declare no competing financial interest.

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