

Enzymatically Driven Remodeling of Growth Kinetics and Internal Structure in Biomolecular Condensates

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Living cells exhibit a complex organization comprising numerous compartments, among which are RNA- and protein-rich membraneless, liquidlike organelles known as biomolecular condensates. Energy-consuming processes regulate their formation and dissolution, with (de)phosphorylation by specific enzymes being among the most commonly involved reactions. By employing a model system consisting of a phosphorylatable peptide and homopolymeric RNA, and thanks to time-resolved ultra-small-angle x-ray scattering enabling us to probe a wide range of length and time scales, we elucidate how enzymatic activity remodels the growth kinetics and the internal structure of biomolecular condensates. Under a passive condition, we reveal a nucleation-driven coalescence mechanism maintained over four decades in time, similar to the coarsening of simple binary fluid mixtures. Coarse-grained molecular-dynamics simulations show that peptide-decorated RNA chains assembled immediately after mixing constitute the basic subunits. In contrast, actively formed condensates initially display a networklike internal structure, which gradually matures upon dephosphorylation prior to coalescence. Both pathways lead to a steady state, but fluorescence recovery after photobleaching indicates a peptide diffusivity that is twice as high in actively formed condensates consistent with their loosely packed internal structure. This study contributes to linking effectively cellular active processes such as post-translational modifications to the formation and dissolution pathways of membraneless organelles, impacting other fields including synthetic biology.

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I. INTRODUCTION

Membraneless organelles are intracellular compartments devoid of lipid membrane, consisting of multiple proteins and possibly nucleic acids (DNA and/or RNA), and formed by liquid-liquid phase separation [1–4]. These biomolecular condensates perform essential biological functions by sequestering specific factors and promoting biochemical reactions following the needs of the cell [5]. They also serve as protocells for the study of the emergence of functional cellular life or as catalytic microcompartments [6,7]. Many condensates found in eucaryotic cells contain RNA and are involved in gene expression, such as nucleoli [8], P granules [9], stress granules [10], or Cajal bodies [11], to name a few. Protein-RNA condensates may also play a role in pathological processes [12], for example by facilitating the transcription of viral RNA dur-

ing SARS-Cov-2 replication [13]. Membraneless organelles are relatively short-lived, in that they are formed to perform specific functions and are dissolved after the completion of these tasks. Such a flexibility is maintained through energy-consuming enzymatic processes. In particular, various post-translational modifications have been shown to regulate intracellular phase separation, including phosphorylation [14], methylation [15], acetylation, and combinations thereof [16].

Phase separation is a phenomenon by which a homogeneous material, spontaneously or not, separates into two or more distinct domains (phases) with different compositions while minimizing the chemical potential. Extensive experimental and theoretical efforts have been devoted to elucidating the phase-separation kinetics of binary systems [17], including fluid mixtures relevant to biomolecular condensates, under both passive (spontaneous) and active (chemically driven) conditions [18–20]. A major limitation in these investigations lies in the lack of information across length and time scales spanning several orders of magnitude. Indeed, biomolecular condensates are mostly micrometer-sized, and therefore fluorescence microscopy has become the standard technique to monitor their lifecycle, especially within cells [8]. Quite recently, holographic microscopy has demonstrated its ability to monitor the growth of PopZ-cation condensates with-

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out the need for protein labeling [21]. In all these cases, submicrometer-sized condensates formed along the growth pathway are overlooked, and events occurring on subsecond time scales often remain inaccessible. Conversely, coarse-grained molecular-dynamics simulations can provide a wealth of information at the molecular level [22], and a few phase-separated protein-RNA condensates have been elucidated [23,24]. Nonetheless, coarse-grained models face substantial challenges in quantitatively reproducing the thermodynamic properties of biomolecular condensates. In addition, although they allow the simulation of relatively large supramolecular systems, the accessible time scales remain limited to a few microseconds at most.

To bridge these multiple scales, time-resolved small-angle x-ray scattering (TR-SAXS) using synchrotron source has emerged as a state-of-the-art technique, enabling us to track self-assembly processes with millisecond temporal resolution and spatial sensitivity ranging from a few to several hundred nanometers [25–29]. Within the context of liquid-liquid phase separation, the assembly kinetics of a prion-like domain has revealed the existence of a double nucleation barrier to overcome prior to condensate growth [30]. With the advent of time-resolved ultra-small-angle x-ray scattering (TR-USAXS) [31] available at a very few synchrotron facilities worldwide, the capabilities of x-ray scattering have extended to the micrometer scale. For example, the spinodal decomposition of bovine serum albumin induced by YCl₃ was probed during the first two minutes following the mixing of components [32]. Likewise, upon mixing oppositely charged polyelectrolytes, mass fractal-like coacervates are formed within milliseconds, which transform to more compact surface fractal morphologies with further growth [33]. Nevertheless, how enzymatic activity remodels the growth mechanism, the internal structure, and the morphology of biomolecular condensates—compared to conditions lacking active processes—remains experimentally unexplored.

To fill this knowledge gap, we employ a model system of biomolecular condensates, the formation of which is triggered by the dephosphorylation of serine residues, thereby mimicking the enzymatic regulation encountered in intracellular membraneless organelles [34]. This system consists of an arginine-rich peptide (RRASLRRASL) containing phosphorylatable serines, associated with poly(uridylic acid) (polyU) RNA. Condensates form spontaneously when the serines bear positive charges—a condition hereafter referred to as passive—or via enzymatically induced by lambda phosphatase (LPP), which removes phosphate groups from initially phosphorylated serines, defining the active condition. This flexible system enables the comparison between condensate formation mechanisms under passive and active conditions. We therefore combine TR-USAXS experiments performed at varying peptide and enzyme concentrations, with confocal microscopy and coarse-grained molecular-dynamics simulations to capture these mechanisms across a wide range of length and time scales.

II. METHODS

A. Materials

The double phosphorylated (RRApSLRRApSL), non-phosphorylated (RRASLRRASL), and N-terminus labeled

5-carboxytetramethylrhodamine (5-TAMRA) forms of the peptide were synthesized by ProteoGenix. Poly(uridylic acid) potassium salt (polyU), approximately 250 nucleotides long (Fig. S1 in the Supplemental Material [35]), HEPES free acid, dithiothreitol, magnesium chloride, manganese chloride, and adenosine 5-triphosphate disodium salt hydrate were purchased from Sigma Aldrich. Lambda protein phosphatase was obtained from New England Biolabs. Poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) was purchased from SuSoS (Switzerland). Buffer, RNA, and peptide stock solutions are prepared using Milli-Q water, and the buffer is subsequently filtered and autoclaved. Condensates are formed by mixing polyU RNA at a fixed concentration of 6.5 μM unless otherwise stated, with various concentrations of peptide ranging from 100 to 2000 μM in a physiological buffer containing 4 mM MgCl₂, 2.25 mM ATP, 1.6 mM dithiothreitol, and 50 mM HEPES pH 7.4 at 37 °C. The samples are thoroughly mixed for at least 1–2 min by pipetting, which allows all components to be evenly distributed.

B. Absorbance measurements

Absorbance measurements are performed using an AvaSpec spectrophotometer equipped with AvaSoft software and a Quantum Northwest TC1 Peltier temperature controller at an absorbance wavelength of 500 nm at 37 °C. For the phase diagram, multiple series of experiments are conducted, varying the peptide concentration from 100 to 1000 μM at various RNA concentrations ranging from 3.5 to 9.5 μM . The samples have a total volume of 500 μL and are incubated at 37 °C for 5 min in a quartz cuvette before measurement.

C. Confocal microscopy

Confocal images are acquired using a Leica TCS SP8 laser scanning confocal inverted microscope equipped with a $\times 63$ oil objective and an H501-T Okolab temperature-controlled stage maintained at 37 °C. The slides and coverslips are treated with O₂ plasma for surface activation and to ensure a sterile environment for the samples. To prevent breakage and excessive spreading of the condensates, microscopic slides and coverslips are incubated with poly(L-lysine)-g-poly(ethylene glycol) (PLL-g-PEG) at a concentration of 0.5 g L⁻¹ in 0.5 mL of 10 mM HEPES buffer pH 7.4 for 30 min, followed by rinsing with HEPES buffer. The PLL-g-PEG coating passivates the glass surface, preventing condensates from adhering to the slide and thereby helping to maintain their spherical morphology. A small fraction ($\approx 0.5\%$) of TAMRA-conjugated peptides is incorporated into the total peptide mixture for fluorescence detection.

The slides are lined with a silicone spacer of 0.24-mm thickness. A total volume of 130 μL of sample is prepared and pipetted on microscopic slides with cavity wells, which have a depth of 0.6–0.8 mm to prevent evaporation during imaging. The samples are then covered with coverslips of 0.17-mm thickness. The excitation/emission wavelengths for TAMRA-peptide are 543 nm/569–589 nm. Time-lapse acquisition experiments are conducted, capturing images every minute over a period of 1–2 h.

All the image analyses are carried out with ImageJ [36].

D. Coarse-grained molecular-dynamics simulations

All molecular-dynamics simulations are performed using OpenMM (v7.5) [37] in the *NVT* ensemble, employing a Langevin integrator with a time step of 10 fs and a friction coefficient of 0.01 ps^{-1} . We use the recently published CALVADOS RNA force field model [38]. In CALVADOS, RNA is modeled as a two-bead-per-nucleotide model potential and includes several components: a bond term U_b , an angle term U_a , an electrostatic term U_{DH} , a short-range Ashbaugh-Hatch [39] (AH) term U_{AH} , and a neighbor stacking term U_{ST} .

The bonded potential is expressed as

$$U_b = \frac{1}{2}k_b(r - r_0)^2, \quad (1)$$

where k_b is the spring constant and r_0 is the equilibrium bond length. These parameters are determined separately for the backbone-backbone and backbone-base distances. For the backbone-backbone bond, the equilibrium length is 0.59 nm with a spring constant of 1400 kJ/mol/nm^2 . For the backbone-base bond, the equilibrium length is 0.54 nm with a spring constant of 2200 kJ/mol/nm^2 .

The angle potential is introduced to account for the stiffness of the backbone chain:

$$U_a = \frac{1}{2}k_a(\theta - \theta_0)^2, \quad (2)$$

where k_a is the spring constant and θ_0 is the equilibrium bond angle. For the backbone chain, $k_a = 4.2 \text{ kJ/mol/nm}^2$ and $\theta_0 = 3.141 \text{ rad}$.

Short-range repulsive and attractive interactions are modeled using the Ashbaugh-Hatch (AH) potential:

$$U_{\text{AH}} = \begin{cases} \epsilon \left[\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^6 \right] + \epsilon(1 - \lambda) & \text{for } r \leq 2^{1/6}\sigma, \\ 4\lambda\epsilon \left[\left(\frac{\sigma}{r} \right)^{12} - \left(\frac{\sigma}{r} \right)^6 \right] & \text{for } r > 2^{1/6}\sigma, \end{cases} \quad (3)$$

where $\epsilon = 0.8368 \text{ kJ/mol}$, and σ and λ are the effective molecular diameter and stickiness parameters, respectively. For interactions between different bead types, the combination rules are applied: $\sigma = (\sigma_i + \sigma_j)/2$ and $\lambda = (\lambda_i + \lambda_j)/2$. The AH potential is truncated and shifted at a cutoff distance of 2 nm. Neighbor stacking interactions are modeled by the AH potential with a smaller σ to mimic strong anisotropic interactions between neighboring beads.

The stacking potential is expressed as

$$U_{\text{ST}} = n_{\text{ST}}U_{\text{AH}}(\sigma_{\text{ST}}), \quad (4)$$

where n_{ST} is the scaling factor (typically larger than unity), and σ_{ST} is smaller than the bead diameter to mimic strong interactions between neighbors. In this case, $\sigma_{\text{ST}} = 0.4 \text{ nm}$ and $n_{\text{ST}} = 15$. The stacking potential is also truncated and shifted at a distance of 2.0 nm, consistent with the AH potential.

Electrostatic interactions are modeled using the Debye-Hückel (DH) potential:

$$U_{\text{DH}} = \frac{q_i q_j}{4\pi\epsilon_0\epsilon_r} \frac{\exp(-r/\lambda_D)}{r}, \quad (5)$$

where q_i and q_j are the charges of the interacting beads (with $-e$ for backbone beads and 0 for base beads), ϵ_0 is the vacuum permittivity, ϵ_r is the dielectric constant of water set to 80, and λ_D is the Debye length. The DH potential is truncated and shifted at a cutoff distance of 4 nm. The Debye length

TABLE I. Peptide composition for simulations mimicking different phosphorylation levels.

System	WT (%)	Mono (%)	Double (%)
S1	0.3	17.1	82.6
S2	13.4	56.0	30.6
S3	25.9	56.0	18.1
S4	43.3	49.3	7.4
S5	71.5	25.8	2.8

was selected to accurately reproduce the experimental phase diagram (ionic strength of 20 mM).

All peptides are modeled using the CALVADOS 2 force field [40]. For peptide-RNA interactions, the combination rules for the AH potential are applied as described above.

Under passive condition, simulations are performed at peptide concentrations of 200, 250, 300, 350, 400, 600, and 800 μM , in the presence of polyU (250 nucleotides) at a fixed concentration of 0.5 mg/mL. All simulations are carried out in a cubic simulation box with a side length of 158 nm. A phase diagram (Fig. S6 in the Supplemental Material [35]) as a function of peptide concentration is constructed by conducting simulations (three replicates per concentration), each initialized from a preformed peptide-RNA condensate. The saturation concentration required for condensate formation is identified to be 350 μM . To investigate the fast dynamics of cluster formation, we select a peptide concentration of 400 μM in combination with 0.5 g/L polyU. A total of 12 simulations are performed starting from a random spatial distribution of peptides and RNA molecules within the simulation box. Each simulation is run for 1 μs (corresponding to 10^8 time steps). For each trajectory, we analyze the number of peptides and RNA chains within the largest cluster (see, e.g., Fig. S8 in the Supplemental Material [35]), defined using a cutoff distance of 1 nm between residues. Three selected replicas are extended up to 30 μs to assess the system's behavior over longer time scales.

To evaluate the effect of phosphorylation on condensate behavior, simulations are performed using the latest version of the CALVADOS force field for phosphorylated disordered protein [41]. Three types of peptides are considered: double phosphorylated (RRApLRRApL), monophosphorylated (RRApLRRASL), and nonphosphorylated/wild-type (WT; RRASLRRASL). For phosphorylated serine residues, charges of both -1 and -2 are tested. Simulations are carried out at a fixed peptide concentration of 800 μM . Each simulation is run for 1 μs . The relative proportions of the three peptide species are varied as detailed in Table I, in order to mimic the time-dependent effect of phosphatase activity. Three replicates are performed for each system. Simulations are analyzed in terms of the number of RNA chains in the largest cluster as a function of time. Cluster geometry is evaluated by computing the gyration tensor $\mathbf{S}$ over all residues belonging to the largest cluster. Asphericity b and radius of gyration R_{gyr} are calculated from the eigenvalues of the gyration tensor ($\lambda_1, \lambda_2, \lambda_3$) as follows:

$$b = \lambda_1 - \frac{1}{2}(\lambda_2 + \lambda_3), \quad (6)$$

$$R_{\text{gyr}}^2 = \text{Tr}(\mathbf{S}) = \lambda_1 + \lambda_2 + \lambda_3.$$

E. Small-angle x-ray scattering

Small-angle x-ray scattering (SAXS) experiments are conducted at the ID02 beamline of the European Synchrotron Radiation Facility (ESRF) in Grenoble (France) at a photon energy of 12.23 keV. The 2D scattering patterns are collected using an Eiger2 4M detector (Dectris, Baden, Switzerland). The data were normalized to an absolute scale after applying different corrections [31] and then azimuthally averaged to 1D intensity profiles $I(q)$, where q is the magnitude of the scattering wave vector and is expressed as $q = (4\pi/\lambda) \sin(\theta/2)$, with λ the wavelength and θ the scattering angle.

The sample-to-detector distance was set to 31 m to explore q -values ranging from 2×10^{-3} to 0.25 nm^{-1} , enabling us to gain more insights into the large-scale structures of biomolecular condensates. For measuring the individual components, the sample-to-detector distance was set to 3 m for RNA and 1 m for peptide, covering a q range of 2×10^{-2} to 2 nm^{-1} . All the measurements were carried out at 37°C .

For static measurements and under active condition, the samples were injected into a flow-through quartz capillary cell with a diameter of 2 mm. For each measurement, around 100 μL of sample solution is thoroughly mixed by repeated pipetting for about one minute and is injected into the capillary. In static, the samples were exposed during 100 ms using an attenuated beam for a total of 40–50 frames with the solution refreshed after each exposure. For kinetics under active condition, 30–60 frames were collected with an exposure time of 100 ms and time intervals of 30 s or 1 min. Radiation damage was not significant in these conditions as measurements performed on peptides and RNA separately with these time sequences have not revealed any change in the scattering patterns. Furthermore, sample dilution due to diffusion is not an issue in flow-through capillary since the sample is in contact with air. The capillary is thoroughly cleaned using Hellmanex, ethanol, deionized water, buffer, and dried using compressed air between different samples. To ensure the samples are washed away from the capillary, the background (buffer) is often measured between samples to make sure it remains consistent with previously measured buffers. Measurements are repeated at least twice to test reproducibility.

For fast kinetics, time-resolved ultra-small-angle x-ray scattering (TR-USAXS) experiments are performed using an SFM-4000 stopped-flow device (Bio-Logic Science Instruments, France) to capture the early times of condensate formation. The mixing quality of the stopped-flow device has been verified by using KBr solution and pure water in various ratios, and by measuring the transmitted x-ray intensity above the K-absorption edge of bromine as a function of time [42]. Stock solutions of RNA, peptide, and buffer are loaded into syringes, and the required final solutions are injected into the stopped-flow capillary cell with a diameter of 1.2 mm at a total flow rate of 4.2 or 6.25 mL s^{-1} , resulting in a dead time of 2.5 ms. For each kinetic measurement, 50 successive frames are captured with an exposure time of 5 ms per frame. The time intervals between frames follow a geometric progression with a ratio of 1.23 and starting with 5 ms. As with flow-through capillary, no radiation damages have been observed on the separate components by measuring them with the same time sequence. Each kinetic measurement is repeated at least

twice to ensure reliability. A total sample volume of about 500 μL per kinetics is injected in such a way that the sample dilution due to diffusion is marginal over the course of the experiment, i.e., typically 15 min.

For both SAXS and TR-USAXS, the scattering intensities were normalized to absolute intensities. Further data processing, such as buffer subtraction and averaging, is performed using SAXSUTILITIES software [43]. TR-USAXS curves are then fitted with a polydispersity sphere model with log-normal size distribution to extract the radius of the condensates using the SASVIEW package [44]. The fits with a polydisperse mass fractal model are carried out with custom-made routines in “MATLAB(R)”.

F. Fluorescence recovery after photobleaching

Fluorescence recovery after photobleaching (FRAP) experiments are conducted using a Leica TCS SP8 laser scanning confocal inverted microscope equipped with a $\times 63$ oil objective and an H501-T Okolab temperature-controlled stage maintained at 37°C . Individual condensates are photobleached for 2 s. A total of 100 frames are captured with a time interval of 77 ms to monitor fluorescence recovery. A total of 15 condensates are analyzed under passive and active conditions.

The confocal images are processed using a custom ImageJ macro that normalizes fluorescence intensity within the bleached region against a control region. The macro extracts mean intensities from user-defined regions of interest (ROIs). The extracted intensity values are plotted over time, and the error-weighted data are fitted with an exponential decay function. The characteristic time τ is extracted from the fit, and the diffusion coefficient D_c is calculated using the formula

$$D_c = \frac{w^2}{4\tau}, \quad (7)$$

where w is the radius of the bleached area.

III. RESULTS

A. Phase separation occurs in the cationic regime

PolyU RNA is estimated to have 250 ± 80 (s.d.) nucleotides based on mass photometry (Fig. S1 in the Supplemental Material [35]). Upon mixing cationic RRASLRASL peptide with anionic polyU RNA [Fig. 1(a)] in 4 mM MgCl_2 , 2.25 mM ATP, 1.6 mM dithiothreitol, 50 mM HEPES pH 7.4 at 37°C , biomolecular condensates in the form of liquidlike droplets are spontaneously formed by coacervation [34]. Direct evidence for the presence of micrometer-sized droplets is given by the turbid aspect of the solution [Fig. 1(b)]. Confocal microscopy images performed with 0.5% of fluorescent TAMRA-peptide confirm the spherical shape and the micrometer-scale size of the condensates [Fig. 1(c)]. Figure 1(d) plots a contour map of the absorbance measured at 500 nm as a function of peptide and RNA concentrations: the highest absorbance values indicate that the incident light is scattered due to the presence of large condensates, while the lowest values are characteristic of dissolved condensates. At a given RNA concentration, absorbance measurements display a clear peptide threshold (saturation) concentration

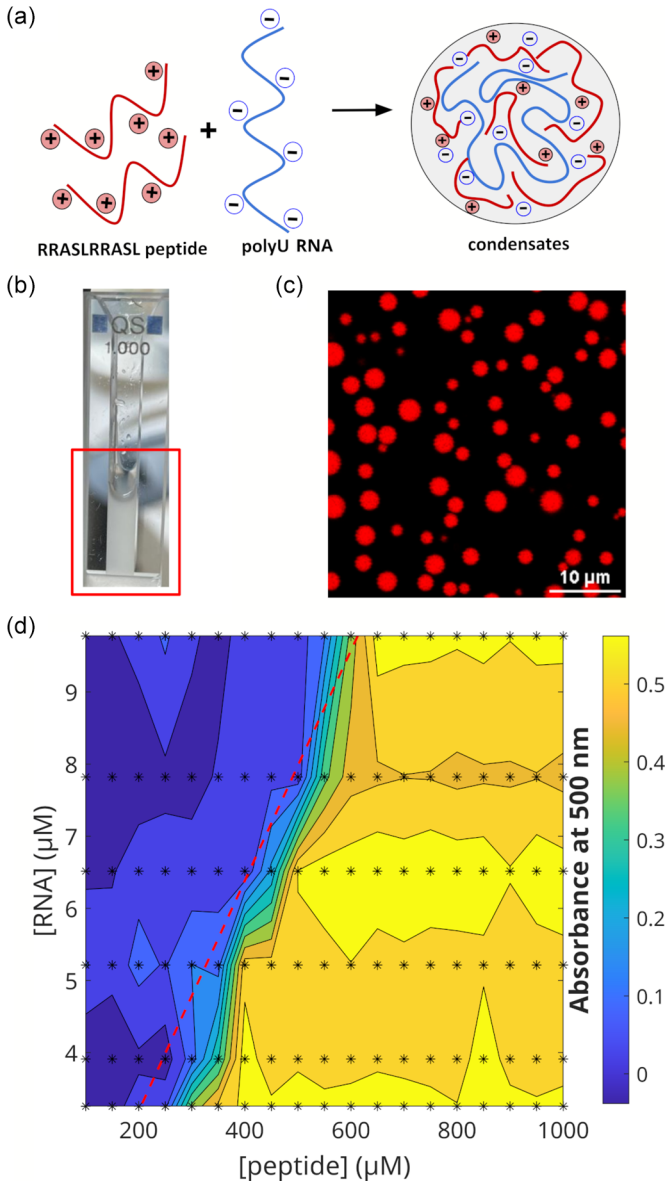


FIG. 1. A phase-separating peptide-RNA system. (a) Schematic representation of RRASLRRASL peptide (red) and polyU RNA (blue) forming condensates upon Coulomb interaction. (b) A solution of condensates in a quartz cuvette exhibits a high level of turbidity. (c) Confocal microscopy image of condensates obtained with 1000 μM of peptide including 0.5% of TAMRA-peptide, and 6.5 μM of RNA. (d) Contour map of absorbance measured at 500 nm as a function of peptide and RNA concentrations. Asterisks show the measured data points, and the red dashed line indicates electroneutrality. PolyU RNA is 250 nucleotides long.

[Fig. 1(d)], which is the hallmark of phase separation. It is worth noting that the phase boundary is almost superimposed with the electroneutrality line. Phase separation occurs in the cationic regime, when the total positive charges supplied by the peptides outnumber the total negative charges carried by RNA.

The SAXS measurement of peptides reveals no sign of aggregation (Fig. S2 in the Supplemental Material [35]) given that the scattering curve at low q -values— q being the magnitude of the scattering wave vector—is flat, which means that the Guinier plateau is reached. Furthermore, the measured radius of gyration ($R_g = 0.9$ nm) is consistent with the small number of amino acids (ten). Likewise, the scattering curve of phosphorylated peptides reveals a radius of gyration of 0.84 nm (Fig. S2 in the Supplemental Material [35]). The radius of gyration of polyU RNA is estimated to be 11.6 nm (Fig. S3 in the Supplemental Material [35]), and the Kratky representation of the RNA scattering curve displays an increase of the normalized intensity ($qR_g)^2(I/I_0)$ at high qR_g —where $I(q)$ stands for the scattering intensity and $I_0 \equiv I(q \rightarrow 0)$ is the forward scattering intensity—which indicates an extended conformation as expected for a fully charged polyelectrolyte.

B. Passively formed condensates grow by nucleated Brownian motion-induced coalescence

Due to the Coulomb interactions underlying coacervation, growth is expected to start as early as a few milliseconds following the mixing of peptides and RNA, spanning length scales from several tens of nanometers to a few micrometers. Accordingly, TR-USAXS experiments are carried out with a very large sample-to-detector distance of 31 m, while peptide, RNA, and buffer solution are mixed rapidly with a stopped-flow device and injected into a quartz capillary for data acquisition with a dead time of 2.5 ms (see the Methods section). Figure 2(a) depicts scattering curves measured at different times for a peptide concentration of 1000 μM (see Fig. S4 in the Supplemental Material [35] for other peptide concentrations). The coacervation begins immediately after mixing and the remarkably flat Guinier plateau at low q -values indicates the structure factor is unity, or, equivalently, the absence of significant interaction between condensates. Note that these measurements are repeatable from the first milliseconds up to nearly 10 min (Fig. S5 in the Supplemental Material [35]).

Since confocal microscopy clearly indicates a spherical morphology for all the condensates [Fig. 1(c)], we fit each scattering curve with a model of noninteracting, homogeneous, polydisperse spheres expressed as (see the Supplemental Material [35] for justification)

$$I(t, q) = I_0(t) \left(\frac{1}{\langle R^6(t) \rangle} \int_0^{+\infty} F_{\text{sphere}}(q; R) p(R; \langle R(t) \rangle, \Delta R(t)) R^6 dR \right) + C_{\text{bkg}}, \quad (8)$$

where R is the condensate radius and $p(R; \langle R(t) \rangle, \Delta R(t))$ is the size distribution assumed here to be log-normal with

mean radius $\langle R(t) \rangle$ and width $\Delta R(t)$. $I_0(t) \equiv I(t, q \rightarrow 0)$ is the forward scattering intensity at each time step. $F_{\text{sphere}}(q; R)$

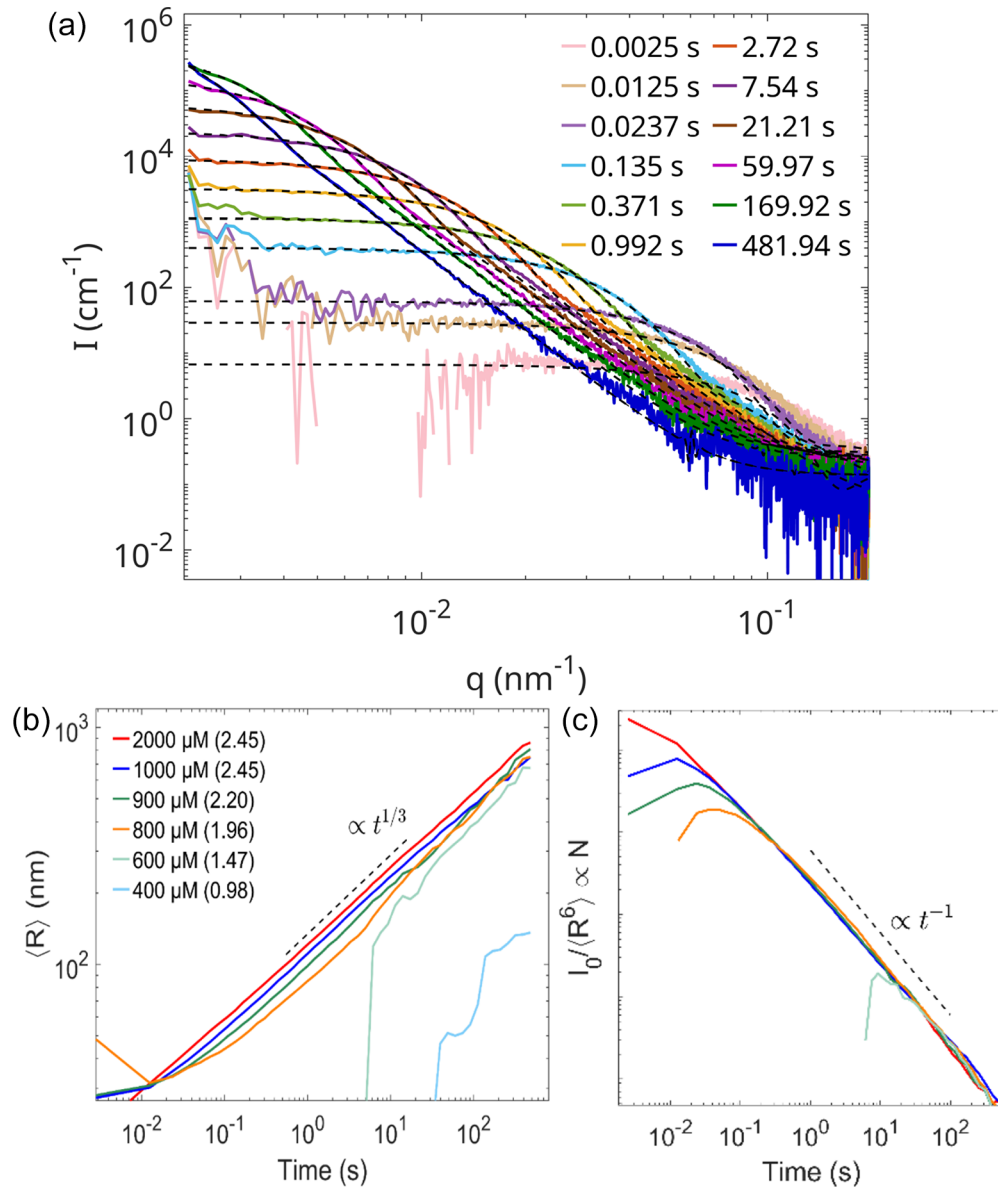


FIG. 2. TR-USAXS measurements under passive conditions analyzed with a model of log-normally-distributed spheres. (a) Scattering curves at different times of a solution containing 1000 μM of peptide and 6.5 μM of RNA (charge ratio: 2.45). The dashed lines are fits with Eqs. (8) and (9). (b) Mean radius $\langle R \rangle$ of condensates as a function of time for various peptide concentrations. The numbers in brackets are the peptide-to-RNA charge ratios. The dashed line represents a $t^{1/3}$ scaling in time. (c) Arbitrarily scaled number density of condensates vs time at various peptide concentrations. The dashed line is a scaling law in t^{-1} . The RNA concentration in (b) and (c) is 6.5 μM except for the highest peptide concentration of 2000 μM , where the RNA concentration is 13 μM .

denotes the form factor of a sphere,

$$F_{\text{sphere}} = \left[3 \frac{\sin(qR) - qR \cos(qR)}{(qR)^3} \right]^2, \quad (9)$$

which verifies $F_{\text{sphere}}(q \rightarrow 0; R) = 1$. $C_{\text{bkg}} [\ll I_0(t)]$ is a constant accounting for the incoherent background intensity. The three fitting parameters at each time step are therefore $I_0(t)$, $\langle R(t) \rangle$, and $\Delta R(t)$. When the mean radius approaches and exceeds $\simeq 1000$ nm, the scattering curves cannot be fitted reliably as the Guinier region moved below the q range of measurement, and accordingly, only data until 482 s are considered.

Figure 2(b) shows the time evolution of $\langle R \rangle$ derived from the scattering curves at various peptide concentrations. Strikingly, the mean radius obeys a power law of the form $\langle R \rangle = (Kt)^{1/3}$ —where K is a dynamic prefactor—as early as the first few tens of milliseconds, and this behavior persists over nearly four decades for the highest peptide concentrations. An exponent of $1/3$ during coarsening in liquid-liquid phase separation is characteristic of either [17,19] (i) diffusion-limited coarsening, or Ostwald ripening, whereby condensate-forming molecules migrate from small condensates to large condensates; or (ii) Brownian motion-induced growth of condensates by coalescence to form increasingly larger condensates. Whether phase separation

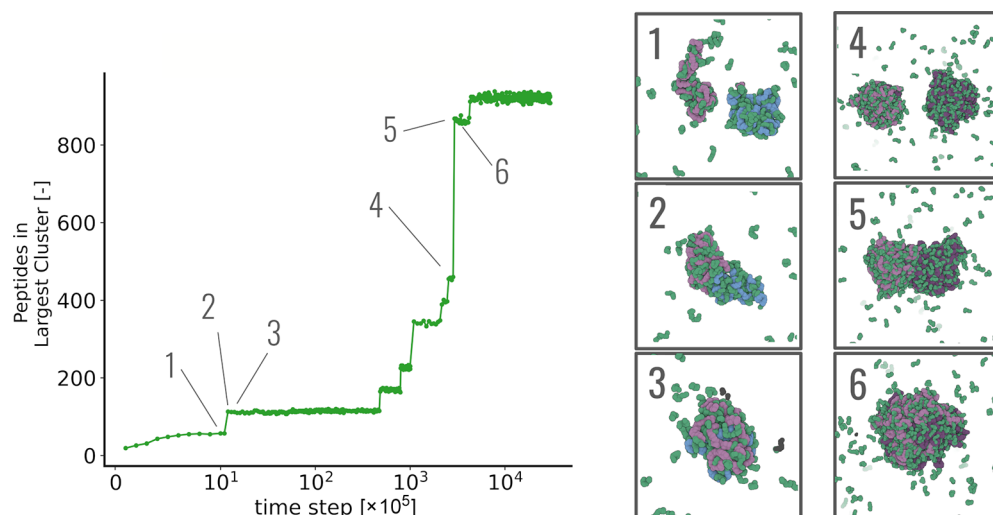


FIG. 3. Long-duration MD simulation of passively formed condensates. The graph plots the number of peptides in the largest cluster as a function of the simulation time steps in logarithmic scale. The snapshots illustrate the coalescence events as labeled on the graph. Peptides (800 μM) are in green and RNA (6.5 μM) in purple (charge ratio: 1.96).

ration is triggered by nucleation or spinodal decomposition, both mechanisms can occur during coarsening and produce a scaling exponent of $1/3$ at the early stage. In the case of coalescence, the dynamic prefactor is expressed as $K \simeq 6k_B T \phi / 5\pi \eta$, with k_B the Boltzmann constant, T the temperature, ϕ the condensate volume fraction, and η the solvent viscosity [19]. Whereas K depends on the condensate volume fraction for the coalescence mechanism, it is only related to the properties of condensate-forming molecules such as the molar volume and the saturation concentration in the case of Ostwald ripening ([45,46]; see Table 1 in [19]). The ratio of the measured dynamic prefactors for experiments performed with peptide concentrations of 1000 and 2000 μM , while maintaining the peptide-to-RNA charge ratio constant, is estimated to be 1.6. Although a factor 2 is theoretically expected, the fact that K increases with volume fraction suggests that coalescence mostly drives the growth kinetics.

A quantity proportional to the number density of condensates N can be deduced from the forward scattering intensity $I_0 \equiv I(q \rightarrow 0)$ and the size distribution at each time point since $I_0 \propto N \langle R^6 \rangle$ [Eq. S4 in the Supplemental Material [35]]. Figure 2(c) depicts this quantity versus time and demonstrates that N scales as t^{-1} , collapsing onto a single curve at late times irrespective of the peptide concentration. This behavior is consistent with a coalescence mechanism as evidenced through a dimensional analysis: the condensate volume fraction ϕ can be approximated as $\phi \propto N \langle R \rangle^3$. Plugging this expression into the scaling law for coalescence, $\langle R \rangle^3 = Kt \propto \phi t \propto N \langle R \rangle^3 t$, leads to $N \propto t^{-1}$, with a prefactor that is independent of ϕ .

Additionally, Fig. 2(b) illustrates the nucleation preceding the condensate growth. As the peptide concentration approaches the saturation concentration ($\simeq 400 \mu\text{M}$), an increasingly long induction time is observed before a sufficient signal-to-noise ratio on the scattering intensity enables us to derive condensate size [see the 400 and 600 μM curves in Fig. 2(b)]. A close examination of the SAXS curves at

400 μM reveals no detectable evolution until $t = 32$ s, while within the following seconds the scattering intensity dramatically increases. We hypothesize that prior to nucleation, each isolated RNA chain is decorated by bound peptides. This interpretation is supported by a comparison between the average scattering intensity measured during induction and the sum of the individual contributions from peptides and RNA. As the former exceeds the latter (Fig. S9 in the Supplemental Material [35]), peptides are indeed bound to RNA. From the forward scattering intensity, we estimate that approximately 30 peptides are bound on each RNA chain (see the Supplemental Material [35]). Furthermore, fitting the average scattering intensity using a Fisher-Burford model (Fig. S9 in the Supplemental Material [35]) [47], which describes simple fractal aggregates, yields a mass fractal dimension of 1.4. This value indicates that the clusters adopt an extended conformation, with a size ($R_g = 13$ nm) comparable to that of bare RNA ($R_g = 11.6$ nm).

To get deeper insights into the early events at the molecular level, we perform coarse-grained molecular-dynamics (MD) simulations with implicit solvent by using a recently released CALVADOS RNA force field [38]. The numerical model is able to reproduce a saturation concentration of 350 μM (Figs. S6 and S7 in the Supplemental Material [35]), that is, within the range of values found experimentally [Fig. 1(d); $\simeq 400 \mu\text{M}$]. Peptides and RNA are initially mixed in a simulation box, and Fig. 3 depicts the time evolution of the number of peptides contained in the largest peptide-RNA cluster. Very rapidly, all the peptides bind on RNA, which are thus decorated with an average of 50 peptides. Remarkably, the total number of bound peptides barely varies afterwards (Fig. S8 in the Supplemental Material [35]), confirming the role of peptide-decorated RNA as stable subunits. The peptide-RNA clusters further grow in a stepwise manner by coalescence. This is illustrated in Fig. 3 wherein the steps observed in the number of peptides in the largest cluster become higher over the course of the simulation. In particular, snapshots 4 and 5 show two clusters comprising 400 and 200 peptides, respec-

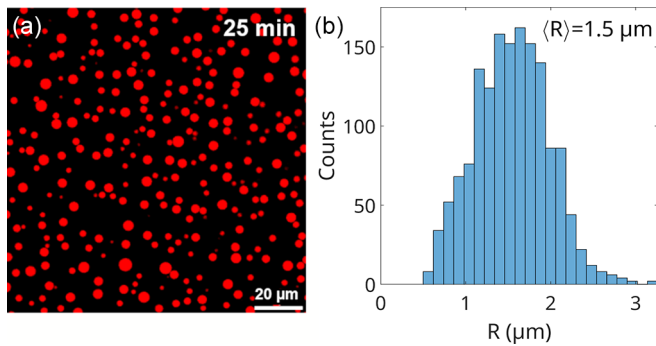


FIG. 4. Passively formed condensates in steady state. (a) Confocal microscopy image of condensates obtained with a peptide concentration of 1000 μM including 0.5% of TAMRA-peptide and 6.5 μM of RNA (charge ratio: 2.45). The image is acquired 25 min after mixing components at 37 $^{\circ}\text{C}$. (b) Condensate radius distribution (total counts: 1534) inferred from a larger view of (a).

tively, that is, eight and four RNA chains (see Replica 1 in Fig. S8 in the Supplemental Material [35]), respectively, merging into a single cluster. Note that fusion events are increasingly rare with the simulation time steps (in logarithmic scale in Fig. 3) as the diffusion of larger clusters is slower. These features, namely the rapid binding of peptides on RNA followed by the condensate growth via coalescence, are robustly observed in independent replicas (Fig. S8 in the Supplemental Material [35]).

To probe condensates of size exceeding several microns, we carried out confocal microscopy imaging by using again 0.5% of fluorescent TAMRA-peptide. Figure 4(a) depicts a low magnification view of condensates at a peptide concentration of 1000 μM , 25 min after mixing with RNA. The radius distribution [Fig. 4(b)] is slightly right-skewed (skewness of 0.19), which is more consistent with the steady-state size distribution theoretically predicted for coalescence than that resulting from Ostwald ripening. Indeed, in the former

case, the distribution is fairly approximated by a log-normal, right-skewed function [19], whereas, in the latter case, the distribution resembles a truncated power law in R^{α} ($2 < \alpha < 3$) and is therefore left-skewed [45,48]. No macroscopic phase separation is observed, most likely as a result of Coulomb repulsion that self-regulates the growth of condensates [21].

C. Networklike internal structure under active condition

In the subsequent series of experiments, we examine the growth kinetics of condensates under active condition. The serine residues of the RRASLRRASL peptide are initially phosphorylated, which results in a neutral net charge. As a consequence, mixing phosphorylated peptides and RNA does not give rise to phase separation. Phase separation is instead triggered by the activity of lambda phosphatase (LPP), which cleaves the phosphate groups from phosphorylated peptides. Figure 5(a) plots scattering curves collected under the same concentrations as in Fig. 2(a) in the presence of 800 U mL^{-1} LPP and using initially double phosphorylated peptide (see Fig. S10 in the Supplemental Material [35] for other peptide concentrations). The condensate formation is much slower than under passive condition because phase separation is now limited by the LPP enzymatic activity. Secondly, the scattering curves in the intermediate q -values, i.e., between 10^{-2} and 10^{-1} nm^{-1} , differ markedly. The inset of Fig. 5(a) presents a comparison between two scattering curves with similar I_0 values. For actively formed condensates, the decrease of $I(q)$ at small and medium q -values is moderate ($I \propto q^{-2.4}$), which suggests a mass fractal structure akin to that found in gels, whereas for passively formed condensates the intensity closely scales as q^{-4} , which is consistent with homogeneous spheres exhibiting a smooth surface. Therefore, provided that condensates are spherical, at least after a certain stage of their formation, as will be evidenced later by confocal microscopy, we opt for a model of polydisperse, spherically symmetric

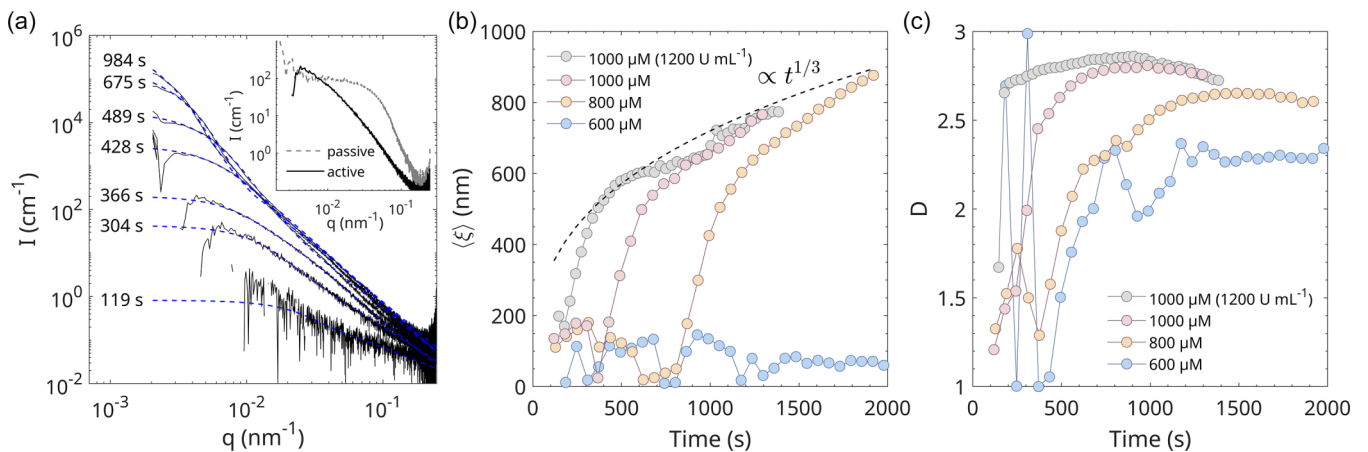


FIG. 5. TR-USAXS measurements under active conditions. (a) Scattering curves at various times (black solid lines) fitted with a polydisperse mass fractal model (blue dashed lines). The solution contains 1000 μM of initially phosphorylated peptide, 6.5 μM of RNA, and 800 U mL^{-1} of LPP. The inset compares two scattering curves collected with peptide and RNA concentrations of 1000 and 6.5 μM , respectively, under passive (gray dashed line) and active (black solid line; 800 U mL^{-1} of LPP), at times where the I_0 values are similar. (b) Mean condensate size $\langle \xi \rangle$ as a function of time for various peptide concentrations. LPP concentration is 800 U mL^{-1} except in one experiment (gray disks; 1200 U mL^{-1}). The dashed line indicates a scaling law in $t^{1/3}$. (c) Mass fractal dimension D vs time for the same experimental conditions as in (b).

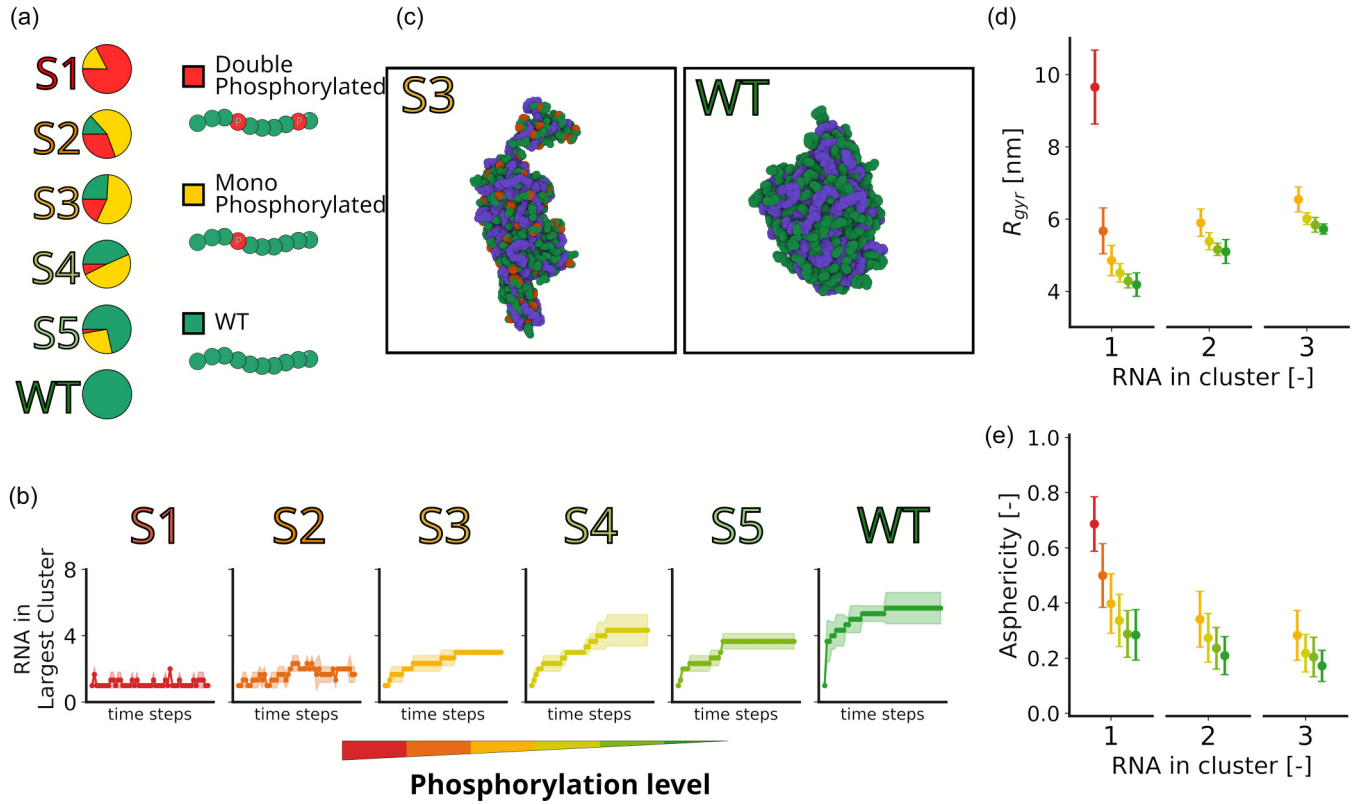


FIG. 6. Coarse-grained MD simulations of condensate growth mimicking active conditions. (a) Peptide compositions of the six simulated systems. (b) Time evolution of the number of RNA chains incorporated into the largest cluster for each system. Shaded areas represent standard deviations over three simulations. (c) Representative snapshots of clusters for the WT and S3 systems. (d) Radius of gyration (R_{gyr}) of clusters containing one, two, or three RNA chains for different peptide compositions. (e) Asphericity of clusters containing one, two, or three RNA chains for different peptide compositions.

clusters, but instead of being homogeneous as previously, they exhibit an internal mass fractal structure parametrized by a fractal dimension [47,49]. The form factor of a mass fractal cluster is expressed by (see the Supplemental Material [35])

$$F_{\text{cluster}}(q; \xi) \propto \int_0^{+\infty} r^{D-1} h(r; \xi) \frac{\sin(qr)}{qr} dr, \quad (10)$$

where D stands for the mass fractal dimension, and $h(r; \xi)$ is a cutoff function that describes the perimeter of the cluster

of size ξ and which must decay faster than any power law. The prefactor ensures the normalization condition, namely $F_{\text{cluster}}(q \rightarrow 0; \xi) = 1$. We choose a generic compressed exponential form for the cutoff function [47], i.e., $h(r; \xi) = e^{-(r/\xi)^\beta}$ with $\beta = 3$, as it best reproduces the scattering curves at late times. As earlier [Eq. (8)], the scattering intensity is computed as a convolution product of the form factor and a log-normal size distribution defined by $p(\xi; \langle \xi(t) \rangle, \Delta \xi(t))$, so that (see the Supplemental Material [35])

$$I(t, q) = I_0(t) \left(\frac{1}{\langle \xi^{2D(t)} \rangle} \int_0^{+\infty} F_{\text{cluster}}(q; \xi) p(\xi; \langle \xi(t) \rangle, \Delta \xi(t)) \xi^{2D(t)} d\xi \right) + C_{\text{bkg}}. \quad (11)$$

In practice, the scattering curves are fitted over the region $q < 0.1 \text{ nm}^{-1}$, which corresponds to length scales above the size of a single RNA chain. The blue dashed lines of Fig. 5(a) represent fits to the experimental data (black solid lines), providing estimates of $I_0(t)$, $\langle \xi(t) \rangle$, $\Delta \xi(t)$, and $D(t)$ for each condition. Figure 5(b) depicts the time evolution of $\langle \xi \rangle$ for three peptide concentrations. We observe mostly two steps: (i) Condensates with size varying around 100 nm are formed from the first minutes after mixing. These condensates display a networklike internal structure as evidenced by a mass fractal dimension D well below 3 [Fig. 5(c)]. After some period

of maturation during which D increases steadily while $\langle \xi \rangle$ remains unchanged, the condensates abruptly start to increase in size, with $\langle \xi \rangle$ seemingly converging toward the classical coarsening behavior scaling as $t^{1/3}$ [black dashed line in Fig. 5(b)]. During the latter stage, D remains stable above 2.5, which indicates that the condensates became more compact. Notice that at 600 μM peptide concentration, no growth is observed for at least the first 2000 s. Increasing the LPP concentration from 800 to 1200 U mL^{-1} [gray disks in Figs. 5(b) and 5(c)] shortens the maturation stage consistently with higher enzymatic activity. Finally, it is worth mentioning that

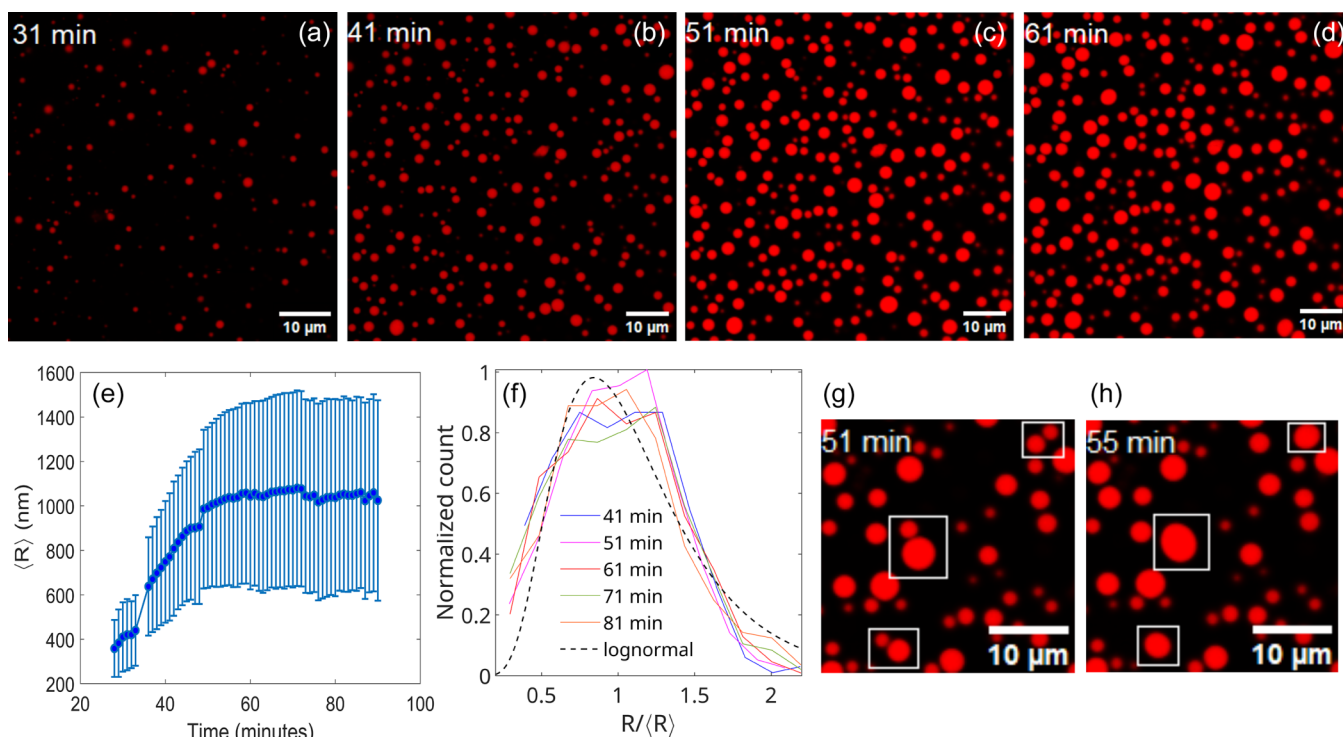


FIG. 7. Confocal microscopy of actively formed condensates. Initial phosphorylated peptide concentration is 1000 μM including 0.5% of TAMRA-peptide, and LPP concentration is 800 U mL^{-1} . (a)–(d) Condensate images acquired at various times after mixing components. (e) Mean condensate radius $\langle R \rangle$ vs time obtained from image analysis. The number of measured condensates is between 120 and 642 depending upon time. Error bars are standard deviations. (f) Normalized size distribution $R/\langle R \rangle$ at various times during growth and steady-state stages. Black dashed line is a log-normal distribution plotted for comparison. (g),(h) Coalescence events between condensates observed at three different locations (white boxes).

although the condensate polydispersity $\Delta\xi/\langle\xi\rangle$ varies significantly during the maturation stage, it tends to a nearly constant value during the growth stage (Fig. S11 in the Supplemental Material [35]), consistent with the self-similar size distribution encountered classically in coarsening mechanisms.

MD simulations that explicitly account for enzymatic activity on phosphorylated peptides are not computationally feasible due to the inherently slow time scales of the reactions involved. Instead, we model cluster formation in systems containing distinct fixed peptide compositions, thereby mimicking different stages of phosphatase-driven dephosphorylation. Figure 6(a) presents the composition of the six simulated systems, ranging from S1, which predominantly contains double phosphorylated peptides, to WT, which consists solely of nonphosphorylated peptides as set under passive conditions. The exact peptide compositions are detailed in Table I. As shown in Fig. 6(b), decreasing the phosphorylation level enhances the ability of clusters to incorporate RNA chains within the same simulation time frame. This behavior is expected because coacervation relies on positively charged peptides to compensate for the negative charges of RNA. More intriguingly, the final clusters display increasingly loose and heterogeneous morphologies when a substantial fraction of double- or monophosphorylated peptides is present, in contrast to the compact aggregates formed by nonphosphorylated peptides [Fig. 6(c)]. Figures 6(d) and 6(e) quantitatively support this observation by showing that both the radius of gyration and the asphericity of the final clusters increase with

peptide phosphorylation level, regardless of the number of incorporated RNA chains. In summary, higher phosphorylation levels lead to less compact and more extended cluster structures, in agreement with experimental measurements.

Next, we investigate the evolution of actively formed condensates over extended length and time scales by confocal microscopy. Figures 7(a)–7(d) depict confocal microscopy images acquired at various times for a peptide concentration of 1000 μM . Figure 7(e) represents the time evolution of the mean radius $\langle R \rangle$ and the standard deviation calculated from image analysis. $\langle R \rangle$ increases until 60 min and remains constant afterwards. Once again, the condensates attain a steady state due to self-regulation driven by Coulomb repulsion. Note that the radii deduced by confocal microscopy [Fig. 7(e)] are underestimated compared to those obtained by x-ray scattering [Fig. 5(b)] as the thresholding step in image analysis trims the edges of small, low-contrast condensates. At a lower peptide concentration of 600 μM , confocal microscopy reveals that 2 h are necessary for the condensate mean radius to reach 1 μm (Fig. S12 in the Supplemental Material [35]). Interestingly, the normalized size distributions, $R/\langle R \rangle$, computed at various times, are mostly superimposed, which supports the self-similarity hypothesized earlier with TR-USAXS data. Additionally, these distributions are slightly right-skewed, resembling the log-normal distribution expected for a coalescence mechanism [19]. This is further illustrated by Figs. 7(g) and 7(h) showing three coalescence events occurring over 4 min.

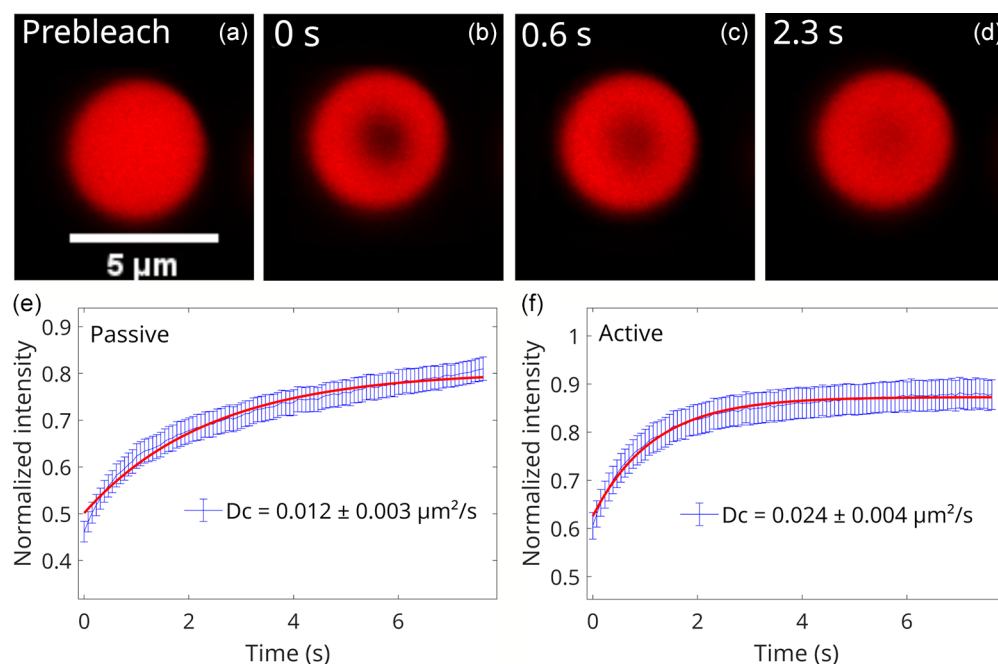


FIG. 8. FRAP measurements on passively and actively formed condensates in steady state. Peptide concentration is $1000 \mu\text{M}$ including 0.5% of TAMRA-peptide. For active condition, 800 U mL^{-1} of LPP is used. (a)–(d) Confocal microscopy images of an actively formed condensate at various times after photobleaching. Normalized fluorescence intensity vs time for passively (e) and actively formed (f) condensates. A total of 15 condensates are used for each condition. Error bars are standard errors of the mean. The diffusivities D_c are deduced by fitting the error-weighted data with an exponential decay function (red line).

D. Actively formed condensates exhibit faster nanoscale dynamics

Intrigued by the difference of internal structures uncovered by x-ray scattering, we carry out fluorescence recovery after photobleaching (FRAP) measurements to estimate the diffusivity of TAMRA-peptide within both passively and actively formed condensates in steady state. Figures 8(a)–8(d) demonstrate the liquidlike nature of condensates with a fluorescence recovery obtained within just a few seconds. By integrating the fluorescence intensity over condensates and fitting the time evolution with an exponential decay function, the diffusivity of TAMRA-peptide is estimated [Figs. 8(e) and 8(f)]. TAMRA-peptide diffuses twice as fast within actively formed condensates ($0.024 \mu\text{m}^2 \text{s}^{-1}$) as within passively formed condensates ($0.012 \mu\text{m}^2 \text{s}^{-1}$). A loosely packed, networklike internal structure as observed under active condition may facilitate a faster translational mobility to TAMRA-peptides, which thereby exhibit an enhanced diffusivity. While other mechanisms cannot be ruled out, it is important to note that TAMRA-peptides are nonphosphorylated, so that their affinity for RNA is identical in both conditions.

IV. CONCLUSION

The kinetics of liquid-liquid phase separation leading to the formation of biomolecular condensates composed of a cationic, phosphorylatable peptide, and polyU RNA—under both passive and active conditions—are compared using multiscale approaches that span up to four decades in time and bridge spatial scales from the molecular to the micrometer

level. Based on the results, Fig. 9 proposes two growth pathways depending on whether enzymatic activity is involved or not.

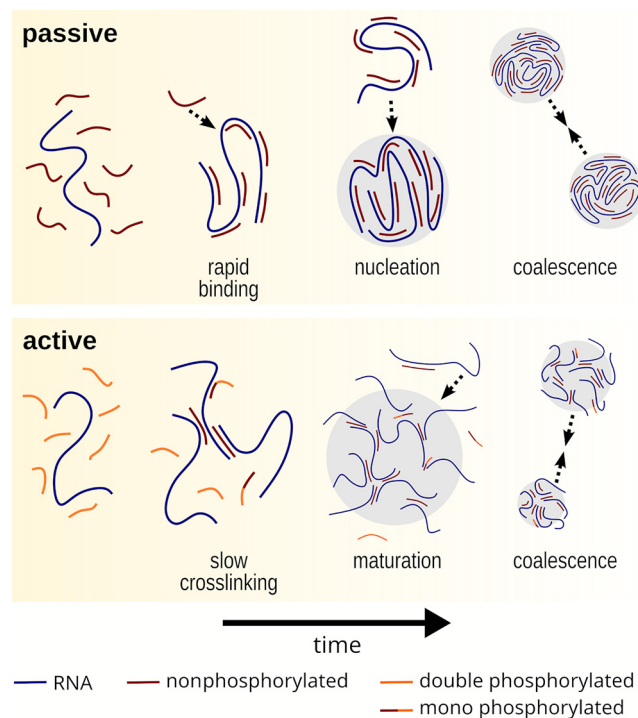


FIG. 9. Schematics of the growth pathways for passively and actively formed biomolecular condensates.

For passively formed condensates, peptides bear a positive net charge and bind on RNA in less than a millisecond after mixing. Peptide-decorated RNA then serve as basic subunits and behave as an immiscible fluid in a solvent. After nucleation, spherical condensates steadily grow following the classical coarsening scaling law in $\langle R \rangle = (Kt)^{1/3}$. The prefactor K , as well as confocal microscopy images and MD simulations, suggest a Brownian motion-induced coalescence mechanism. Eventually, coalescence is impaired by Coulomb repulsion, leading to a steady state. Coalescence is preferred over ripening likely because transferring large peptide-decorated RNA from small to large condensates would be too costly in terms of free energy.

As for actively formed condensates, double phosphorylated peptides bear a zero net charge and bind on RNA upon phosphate cleavage by LPP. Resultantly, RNA chains are slowly cross-linked by dephosphorylated peptides making up loosely packed condensates with a networklike internal structure. Recall that a double phosphorylated peptide bears two serines, each of which carries one phosphate group cleavable by LPP independently of the other. Under the action of LPP, peptides can be nonphosphorylated (two phosphate groups cleaved) or monophosphorylated (only one phosphate group cleaved). Nonphosphorylated peptides, however, are required to trigger phase separation [34]. As dephosphorylation proceeds, more RNA chains are incorporated into condensates, which concomitantly become more compact. After some maturation period, perhaps once nonphosphorylated peptides are supersaturated, condensate size abruptly increases upon coalescence, until the steady state is reached.

Elucidating the nonequilibrium dynamics of biomolecular condensates is essential for understanding the functions and the pathological significance of membraneless organelles in the cell, as well as designing synthetic liquidlike carriers for intracellular delivery of therapeutic mRNA for instance [50]. Even though the phase diagram of multicomponent fluid mixtures is inherently complex, particularly in the presence of charged polymers [51–53], the growth kinetics of our passively formed condensates obey the simple droplet coarsening law ($\langle R \rangle \propto t^{1/3}$) for binary mixtures. Introducing enzymatic activity delays the onset of the reaction-limited growth, but more importantly, kinetic effects modulate the internal struc-

ture of condensates. A loosely packed content may be crucial for preventing dynamic arrest, which can occur with the highly charged biomolecules found in the cell nucleus [54]. In that sense, enzymatic activity may not only regulate the formation and dissolution of biomolecular condensates, but also the viscoelastic properties that govern nanoscale dynamics [54,55].

A recent study has reported that kinetics govern the large-scale spatial organization of phase-separated DNA droplets [56]. In particular, fast condensation was found to promote the formation of hyperuniform structures. Although our passively formed condensates grow on a faster time scale, we find no clear evidence of hyperuniformity, neither on scattering curves nor on confocal microscopy images (Fig. S13 in the Supplemental Material [35]). Therefore, other factors that govern the large-scale spatial organization of phase-separating fluids are yet to be identified.

At last, a number of condensates, notably transcriptional condensates, are immersed in a crowded environment that can be modeled as a heterogeneous elastic medium. Recent theoretical advances have uncovered a rich diversity of condensate growth kinetics governed by local environmental stiffness [57]. Investigating these effects experimentally using model condensates will be an important direction for future research.

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DATA AVAILABILITY

There are no publicly available research data or software supporting this manuscript. Requests for further information or data should be sent to the authors.

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