Kinetic interplay between droplet maturation and coalescence modulates shape of aged protein condensates

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Biomolecular condensates formed by the process of liquid–liquid phase separation (LLPS) play diverse roles inside cells, from spatiotemporal compartmentalisation to speeding up chemical reactions. Upon maturation, the liquid-like properties of condensates, which underpin their functions, are gradually lost, eventually giving rise to solid-like states with potential pathological implications. Enhancement of inter-protein interactions is one of the main mechanisms suggested to trigger the formation of solid-like condensates. To gain a molecular-level understanding of how the accumulation of stronger interactions among proteins inside condensates affect the kinetic and thermodynamic properties of biomolecular condensates, and their shapes over time, we develop a tailored coarse-grained model of proteins that transition from establishing weak to stronger inter-protein interactions inside condensates. Our simulations reveal that the fast accumulation of strongly binding proteins during the nucleation and growth stages of condensate formation results in aspherical solid-like condensates. In contrast, when strong inter-protein interactions appear only after the equilibrium condensate has been formed, or when they accumulate slowly over time with respect to the time needed for droplets to fuse and grow, spherical solid-like droplets emerge. By conducting atomistic potential-of-mean-force simulations of NUP-98 peptides—prone to forming inter-protein β-sheets—we observe that formation of inter-peptide β-sheets increases the strength of the interactions consistently with the loss of liquid-like condensate properties we observe at the coarse-grained level. Overall, our work aids in elucidating fundamental molecular, kinetic, and thermodynamic mechanisms linking the rate of change in protein interaction strength to condensate shape and maturation during ageing.

Living cells contain numerous macromolecular components, which must be organised in space and time to facilitate the concerted regulation of biochemical reactions1–4. In eukaryotes, such functional organisation is achieved via the formation of both membrane-bound5 and membrane-less compartments6–8. The latter, also termed biomolecular condensates7, are liquid drops of varying compositions thought to form via liquid–liquid phase separation (LLPS) when a critical concentration of key multivalent biomolecules (such as proteins and RNA) is surpassed2,6. Biomolecular condensates are ubiquitous within both the cytoplasm7 and nucleusplasm8–12, with the most well-known examples including P granules13, nucleoli14–19, Cajal bodies20–22, or stress granules23. Biomolecular condensates have also been identified as functional organisers of the interiors of prokaryotes24.

Intracellular LLPS is a delicate phenomenon which is sensitively affected by the environmental conditions (e.g., pH, salt, and temperature)25,26, and the presence of different molecular partners15,27,28. Alteration of such conditions can lead to misregulation with pathological implications29–31. Indeed, the gradual rigidification of biomolecular condensates with time (also known as ‘maturation’ or ‘ageing’) has been associated to the proliferation of multiple neurodegenerative diseases29–33—such as amyotrophic lateral sclerosis (ALS)34, Parkinson’s35, Alzheimer’s36, and frontotemporal dementia (FTD)—and of certain types of cancers37 and diabetes38. Therefore, understanding the molecular mechanisms influencing aberrant LLPS is a key area of biomedical research39.

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Macroskopically, biomolecular condensates present liquid-like properties, such as the ability to coalesce and deform under shear flow, exhibit spherical shapes, show short recovery times from fluorescence recovery after photobleaching (FRAP) or GFP florescence recovery experiments, and exchange material rapidly with their environment. Macroscopically, such liquid-like properties originate from the weak multivalent attractive interactions that the biopolymers within the condensate establish. Weak interactions translate into dynamic binding and unbinding, free molecular diffusion within, and facile exchange of species in and out of condensates. Overall, the liquid-like behaviour of molecules enables condensates to fulfill a wide-range of biological functions, from acting as curated reactive volumes that selectively concentrate and exclude specific molecules, buffering of protein concentrations, regulating gene expression, sensing changes in the cell environment, to sequestering components harmful in the cell.

Although the liquid-like properties of condensates seem to underpin their functions during health, it is now clear that the material properties of condensates extend far beyond those of low viscous liquids. Indeed, condensates encompass low to high viscosity fluids, hydrogels and solid-like states. These properties are not surprising if one considers that the physicochemical features of the biopolymers known to form condensates are highly heterogeneous too. These include multidomain proteins, intrinsically disordered regions (IDRs), and globular proteins with different chemical makeups, and which can undergo LLPS in pure form via homotypic interactions and/or in partnership with other proteins, RNAs, DNA, or chromatin via heterotypic interactions. Furthermore, FRAP, GFP florescence recovery, coalescence, and active and passive microrheology experiments have revealed that over time, even the condensates that are originally liquid-like can transition to gels or soft glasses upon maturation. Matured condensates display reduced fusion propensities and longer recovery times after photobleaching, which suggest that the diffusion of molecules within is significantly reduced. Several factors have been proposed as key drivers for the liquid-to-solid transition of condensates including altered solvent concentrations, post-translational modifications, protein mutations, and protein folding and misfolding events. All these factors are expected to favour rigidification by increasing the binding affinity among species and slowing down the timescales of inter-protein unbinding events.

In this work, we develop a coarse-grained (CG) simulation approach to investigate the impact of the gradual strengthening of inter-protein interactions—due for instance to the accumulation of inter-protein \( \beta \)-sheets, post-translational modifications, or changes in the condensate microenvironment—in the kinetics and stability of protein condensates over time. Our CG simulations reveal the interplay of the timescales of condensate growth and fusion, and the rate of emergence of stronger inter-protein interactions, critically dictates condensate shape: with spherical condensates forming when fusion dominates, and aspherical solid-like states arising when the stronger interactions accumulate faster than the timescales of condensate fusion. Finally, using atomistic simulations, we show that formation of inter-protein \( \beta \)-sheets can strengthen interactions sufficiently to trigger the type of dynamical arrest of condensates we observe at the coarse-grained level. Taken together, our simulations provide a time-dependent assessment of the modulation of the dynamic properties of proteins inside condensates, and contrast kinetics and thermodynamics properties of condensates sustained by strong versus transient inter-protein interactions.

### Results and discussion

#### Strengthening of inter-protein interactions can cause condensate maturation and thermal hysteresis.

We begin by investigating how strengthening of inter-protein interactions affects the thermodynamic and rheological properties of condensates. For this purpose, we develop a tailored coarse-grained model that can assess the impact of transient versus long-lived protein binding on the kinetic and thermodynamic properties of the condensates they form. Our model approximates an intrinsically disordered protein as a fully flexible Lennard-Jones heteropolymer of beads connected by harmonic springs (see Fig. 1A, and section SI). Each bead represents a protein binding region that corresponds to a linearily consecutive group of around six to eight amino acids. Such bead resolution is arbitrarily defined to represent the smallest protein regions that undergo disorder-to-order transitions, or that can accumulate sufficient post-translational modifications to result in significant strengthening of inter-protein interactions. We set each heteropolymer to contain 39 beads, or 234–312 amino acids, which is well within the typical length of many intrinsically disordered protein regions of intracellular phase separating proteins (e.g. hnRNPA1 IDR, FUS PLD, LAF-1 IDR, DDX4 N-terminal domain). Within a single heteropolymer, we combine beads representing two different types of ‘sticker’ regions (labelled A and B beads) prone to establishing strong heterotypic interactions with their complementary stickers (i.e., A–B pairs), and beads representing ‘spacer’ regions that only establish weak interactions (Fig. 1A). Specifically, we distinguish two types of possible interactions among beads: (1) weak interactions for any sticker–spacer and spacer–spacer pair, and for homotypic sticker–sticker pairs. Pairs of weakly (i.e., interaction strength equal to \( \varepsilon_S \)), and (2) strengthened interactions only among complementary pairs of 'sticker A–sticker B' beads (i.e., 10 times stronger or equal to \( \varepsilon_S = 10\varepsilon_B \)) (Fig. 1A). The latter restriction of only considering strong interactions among pairs of complementary A–B stickers, rather than among all stickers, is set to implicitly consider that stronger interactions would most likely be favoured among sticker regions that are not only in spatial proximity but also favourably orientated with respect to one another. The stickers-vs-spacers sequence patterning of vs. weak) of our coarse-grained proteins is shown in Fig. 1A and SIA (Top). Moreover, an alternative patterning for strong versus weak interactions in which beads representing sticker domains are only located along the first half of the coarse-grained sequence (Fig. SIA (Bottom)) is also explored in the SI to elucidate possible patterning effects in condensate maturation. The comprehensive description of the coarse-grained potentials and a full list of the model parameters, as well as protein sequences and the employed reduced units are provided in Sections SIA and SIB of the Supplementary Information.
As a control, we begin by characterising the dynamical properties of proteins inside condensates in the absence of strengthen interactions. In such a homopolymer model, a value of the bead–bead interaction strength, $\varepsilon_D$, larger than $0.35k_B T$ enables the formation of phase-separated droplets. Therefore, we perform unbiased Molecular Dynamics (MD) simulations of roughly 730 interacting homopolymers proteins in the NVT ensemble, where all beads bind to one another with a uniform binding strength equal to $\varepsilon_D = 0.66 k_B T$; such value of $\varepsilon_D$ is high enough to induce condensate formation. From bulk simulations at the equilibrium condensate density, we estimate the mean square displacement (MSD) of the central bead of each protein (in $\sigma$ units, the molecular diameter of every bead in our model), and calculate the value of the diffusion coefficient ($D$) of proteins within the condensates as a function of time (in reduced units $\tau^*$) (Empty blue triangle of Fig. 1B; for further details on these calculations see SIB of the Supplementary Information). We observe that the diffusion coefficient of proteins within the droplets quickly converges reaching a value of $\sim 0.002 \sigma^2/\tau^*$, characteristic of the free diffusion of polymers within liquids.
We next investigate the change in the mobility of the proteins within condensates when long-lived binding due to strengthening of inter-protein interactions occurs. To do so, we use our heteropolymer model, where now 34 beads are treated as spacer regions (i.e., bind to one another weakly with $\varepsilon_D$) and 5 beads are treated as sticker regions (i.e., bind to most regions weakly with $\varepsilon_D$, but to complementary sticker regions strongly with $\varepsilon_S$). Note that the value of $\varepsilon_D$ controls the strength of interactions among both weakly and strongly binding regions ($\varepsilon_D = \varepsilon_S/10$). Since the strengthening of inter-protein interactions would depend on the sequence of the amino acids involved\(^{79,82}\) and the physicochemical factors driving such strengthening (e.g. disorder-to-order transitions\(^{74-76,79,80}\), post-translational modifications\(^{81}\), or changes in salt conditions\(^{80}\)), we explore the dependence of the changes in protein diffusion within condensates on the relative binding interaction strength among beads. Given that values of $\varepsilon_D$ larger than 0.35 $k_BT$ enable the formation of phase-separated droplets, we vary $\varepsilon_D$ from 0.5 to 0.66 $k_BT$, and $\varepsilon_S$ correspondingly (Fig. 1B Left). These tests reveal that when proteins bind to one another weakly ($\varepsilon_D = 0.5 k_BT$ and $\varepsilon_S = 5 k_BT$, orange curve), the average diffusion coefficient of proteins within the droplets decays moderately due to the emergence of small clusters of strong inter-protein contacts. The diffusion coefficient then quickly plateaus at a sufficiently high value—signalling ergodic liquid-like behaviour. In contrast, at stronger protein interaction strengths ($\varepsilon_S \geq 5.25 k_BT$, magenta and red blue curves), the diffusion coefficient decays significantly and now fails to reach a plateau within the explored simulation timescale. Note that to measure $D$ over time, we choose sufficiently large windows of time that allow the central bead of the proteins to diffuse distances at least 3–5 times their molecular diameter; over time, due to the deceleration of the protein mobility, these windows need to be expanded to longer timescales to fulfill the required length scale of sampling. The observed behaviour of the diffusion coefficient signals a significant and continuous decay in the protein mobility, consistent with progressive condensate maturation\(^{23,34,49,65}\). Moreover, the emergence of strong binding domains results in a moderate gradual densification of the droplets (Fig. S2). Such condensate densification, as well as the reduction in protein mobility, are driven by the gradual accumulation of strong intermolecular interactions (Fig. S3); this is in contrast to the quick equilibration of the diffusion coefficients in our simulations, where we treated proteins as weakly-binding homopolymers (even at a value of $\varepsilon_D = 0.66 k_BT$, blue empty triangle). Decreased mobility of proteins over time, leading to aged condensates (i.e., the ‘ageing regime’), has been inferred experimentally from decelerated diffusion coefficients, higher condensate viscosities\(^{35,83}\), and lower or incomplete recovery from photobleaching\(^{29,34,35,65-69,84}\). Moreover, from Fig. 1B we can observe how the protein diffusion coefficient within the condensates is highly sensitive to small variations in the binding strength between domains. That is, the diffusion coefficient decreases by several orders of magnitude when the binding strength among domains ($\varepsilon_S$) is raised from 5 to 6.6 $k_BT$.

Our simulations reveal that there is a clear inter-protein interaction strength threshold that separates ergodic liquid-like behaviour from non-ergodic ageing behaviour towards glassy droplets ($\varepsilon_S > 5 k_BT$), which we depict by a horizontal black dashed line in Fig. 1B (Left panel). Above such threshold, condensates readily equilibrate and form spherical droplets within the accessible simulation timescales. Below this threshold, condensates gradually become kinetically trapped, forming amorphous droplets due to the emergence of long-lived interactions that hinder the diffusion of proteins within (Fig. 1B Right panel). These independent simulations further support the location of the kinetic threshold shown in Fig. 1B (Left panel). The timescale for the onset of strong binding between protein regions during nucleation and growth of the condensates, significantly impacts condensate shape (Fig. 1B Right panel). As expected, condensates that emerge from proteins that bind to one another weakly (i.e., $\varepsilon_S \leq 5 k_BT$) grow into spherical liquid droplets. Spherical shapes are favoured because they minimize the surface-to-volume ratio and the interfacial free energy cost within the dilute phase and under the specific imposed box dimensions and number of protein replicas\(^{85}\). However, we note that for other box dimensions with higher global densities, slabs and cylindrical droplets can also minimize the surface-to-volume ratio and the interfacial free energy of the system\(^{86}\). Nevertheless, in our box system sizes, the simulations of homopolymers proteins (i.e., where all residues bind to one another evenly) can form both spherical condensates at low to moderate values of the protein–protein binding strength (from $\varepsilon_D > 0.4 k_BT$ to $\varepsilon_D < 0.9 k_BT$; Fig. S8 (Top panel)) and amorphous kinetically-arrested condensates at higher values ($\varepsilon_D > 1 k_BT$) (see almost instantaneous quenching in protein diffusivity in Fig. S8 (Bottom panel)). We find that condensates resulting from heteropolymer proteins that contain both weakly binding spacers, and stickers that bind to complementary stickers more strongly (i.e., $\varepsilon_S \geq 5.25 k_BT$), always give rise to aspherical kinetically-arrested condensates (Fig. 1B Right Bottom panel). In this case, the emergence of longer-lived interactions prevents individual proteins from relaxing and conveniently rearranging within the condensate to minimise the surface tension, and thus, their free energy\(^{87,88}\). We also note that a qualitatively similar behaviour is obtained when the strongly interacting sticker beads are placed at the first half of the sequence (Fig. S4) rather than distributed over its full length (Fig. 1B). Only a moderate increase of the inter-protein interaction strength threshold ($\varepsilon_S \geq 6 k_BT$) respect to that shown in Fig. 1B ($\varepsilon_S \geq 5.25 k_BT$) is required to switch from ergodic liquid-like behaviour to transient ageing behaviour (Fig. S4). Similarly, when we use the Wang–Frenkel potential\(^{89}\) to model bead–bead non-bonded contacts, which significantly reduces the range of strong interactions (Fig. S1B pink curve), we find that just a minor increase of $\varepsilon_S$ to values $\geq 6 k_BT$ is needed to bring condensates from a liquid-like state into the ageing regime (Fig. S6). If we assume that a standard protein diffusion coefficient of an intrinsically disordered protein within a phase-separated condensate is of the order of $\sim 1 \mu m^2/s$ (Ref.\(^7\)), we can estimate that the average time for a single protein to diffuse a typical distance within a condensate (i.e., 5 $\mu m$) is about 4 s. Hence, a protein diffusion deceleration of about 2–3 orders of magnitude, as the one we found in our simulations (Fig. 1B), would imply that proteins within aged condensates would require from 5 min to 1 h to migrate the same distance that takes them seconds inside a liquid-like droplet\(^{88}\). These relative timescales extracted from our simulations are consistent with the observed behaviour in time maturation experiments and FRAP recovery experiments applied to multiple aged condensates\(^{23,34,65}\).
We also study the thermal hysteresis of matured condensates. To that end, we set a protein interaction strength that enables liquid-liquid phase separation at $T = 300\, \text{K}$ ($\varepsilon_S = 5\, \text{k}_B\, T$). From the initial homogeneous system, proteins nucleate several small spherical droplets that grow and coalesce eventually yielding a single spherical larger condensate (i.e., the global free energy minimum in the liquid-like regime). By starting from the same homogeneous system, we then increase the interaction strength among proteins to $\varepsilon_S = 6.6\, \text{k}_B\, T$ (ageing regime) in order to promote the formation of stronger longer-lived interactions. As expected from Fig. 1B, we observe the emergence of an amorphous elongated kinetically-arrested condensate (Fig. 2A (Bottom)). Snapshots of the condensate shape as a function of time are shown. Protein segments that do not participate in strengthen contacts are depicted in grey, while those involved in clusters of stronger interactions are coloured in green. The protein interaction strength of this simulation was set to $\varepsilon_S = 6.6\, \text{k}_B\, T$, the same set value for the condensates shown in the bottom left panel of Fig. 1B.

**Figure 2.** (A) Thermal hysteresis of the condensates probed via coarse-grained protein simulations. (Top panel) Time-evolution starting from an homogeneous system where inter-protein interactions are moderate (i.e., $\varepsilon_S = 5\, \text{k}_B\, T; \varepsilon_D = 0.5\, \text{k}_B\, T$). (Bottom panel) Time evolution at the same conditions above, although starting from a matured condensate that was formed under ageing regime conditions (i.e., strong inter-protein interactions of $\varepsilon_S = 6.6\, \text{k}_B\, T$). Note that in our model, temperature $T$ is proportional to $\frac{1}{\varepsilon_D}$ (B) Number of strong interactions as a function of time within a preformed spherical condensate ($N_{s-s}$) normalised by the typical strong contact threshold (horizontal dashed line) that induces ageing behaviour of protein condensates at those conditions (i.e., number of strong interactions per condensate volume found at the cross-over of the blue curve with the kinetic threshold shown in Fig. 1B). Snapshots of the condensate shape as a function of time are shown. Protein segments that do not participate in strengthen contacts are depicted in grey, while those involved in clusters of stronger interactions are coloured in green. The protein interaction strength of this simulation was set to $\varepsilon_S = 6.6\, \text{k}_B\, T$, the same set value for the condensates shown in the bottom left panel of Fig. 1B.
These results further demonstrate how droplet shape can be critically modulated by the competition between two distinct timescales: coalescence time and maturation rate. This behaviour is particularly well exemplified...
Moreover, we note that different patterning of strong-binding domains along the protein sequence (Figs. S4)
fusion events of kinetically arrested droplets rather than from maturation of preformed spherical condensates. The blue curve is a kinetic line that is defined as the intersection of the different diffusion curves and the horizontal kinetic threshold shown in Fig. 1B (Left panel). Filled squares represent the time required for two spherical tangent droplets of a given size to fuse into a single spherical condensate, while empty squares depict the (arbitrary) maximum simulated time for tangent droplets that did not achieve complete coalescence or shown strong trends of the formation of a single spherical condensate. Snapshots of the typical time-evolution of coalescing droplets in both regimes at $\varepsilon_S = 5.25 k_B T$ are included for droplet sizes of 100 (liquid-like regime) and 200 proteins (ageing regime).

by the simulations at inter-protein interaction strengths just sufficiently high to give rise to arrested glass-like behaviour (i.e., $\varepsilon_S = 5.25 k_B T$). In this case, for the smallest droplet sizes (50 and 100 proteins per droplet, purple and green squares respectively in Fig. 3), the time required for droplet fusion and minimisation of the system’s free energy (i.e., forming a spherical condensate) is shorter than the maturation time (i.e., the formation of long-lived interactions that cause the system to become kinetically trapped). However, for larger droplet sizes (i.e., those containing 200 and 500 proteins), the condensate (both tangent droplets) becomes kinetically arrested before achieving a spherical arrangement. On the other hand, moderate inter-protein interactions permit the complete coalescence of all tested condensate sizes into spherical droplets ($\varepsilon_S = 5 k_B T$), while stronger interactions (i.e., $\varepsilon_S = 5.75 k_B T$) do not yield complete fusion of even the smallest tested droplets (Fig. 3). We note that the quantitative absolute values of the kinetic and thermodynamic magnitudes measured in our simulations are determined by the specific features of our models and the implicit treatment of the solvent. Nevertheless, the qualitative relative trends that we observe for these magnitudes (i.e., $D$ or $\rho$) over time, and the interplay between droplet fusion rate, protein binding strength and protein mobility (measured in Fig. 3) are expected to hold. For instance, having implicit solvent overestimates protein self-diffusion within the condensates, but also increases the droplet fusion rate of the two tangent droplets. In addition, while protein diffusion coefficient are likely overestimated, we are neglecting the small free energy barriers associated to the emergence of strong-binding domains that are accomplished through disorder-to-order structural transitions\(^{108}\). Overall, our simulations highlight how small variations in the binding energy between protein domains can crucially modulate the liquid-like behaviour, and ultimately the shape of biomolecular condensates.

Based on Figs. 2 and 3, we argue that condensate asphericity seems to be fundamentally determined by fusion events of kinetically arrested droplets rather than from maturation of preformed spherical condensates. Moreover, we note that different patterning\(^{29}\) of strong-binding domains along the protein sequence (Figs. S4 and S5), does not show a qualitatively distinct behaviour to that of Fig. 2 in terms of shape evolution along condensate maturation\(^{29}\).

**Comparing the strength of inter-protein interactions among disordered versus ordered peptides.** In this section, we quantify the change in the strength of inter-protein interactions due to the formation of inter-protein $\beta$-sheets, to determine if such change may be consistent with the dynamical arrest we describe in our coarse-grained simulations. We are particularly interested in the formation of inter-protein $\beta$-sheets because they can emerge spontaneously and intrinsically, i.e., without requiring changes in the chemistry of the system or the environmental conditions. Interestingly, the intrinsically disordered regions of various phase-separating naturally occurring proteins—including fused in sarcoma (FUS)\(^{75}\), TAR DNA-binding Protein of 43 kDa (TDP-43)\(^{36}\), heterogeneous nuclear ribonucleoprotein A1 (hnRNPA1)\(^{74,79,80}\), and nucleoprotein of 98 kDa (NUP-98)\(^{34,109}\)—which form hydrogels over time\(^{3,116,111}\), contain short regions termed Low-complexity Aromatic-Rich Kinked Segments (LARKS) that are prone to form such inter-protein $\beta$-sheets\(^{82}\). When multiple LARKS meet at the high concentrations found inside condensates, they can assemble into ordered arrays of inter-protein $\beta$-sheet structures that stick to one another strongly via $\pi-\pi$ bonds and hydrogen bonding between backbone atoms that may lead to gradual solidification of, otherwise, liquid-like condensates\(^{72,74,75,77,87,112}\). Importantly, hundreds of protein sequences capable of such disorder-to-order conformational transitions, and concomitant enhancement of intermolecular binding strengths, have been identified in the human genome\(^{24}\).
As a case study, we focus on the NUP-98 protein—an aggregation-prone protein that phase separates in vitro under selective conditions and can form hydrogels under others\cite{13,14}. We start by estimating the binding strength among four interacting NUP-98 LARKS-containing peptides by means of Umbrella Sampling Molecular Dynamics simulations\cite{15} in explicit solvent and ions under two distinct scenarios: (1) when all the peptides are fully disordered, and (2) when peptides form the inter-peptide cross-$\beta$-sheet motif resolved crystallographically (PDB code: 6BZM)\cite{74}. From these simulations (using the a99SB-disp force field\cite{116}), we compute the potential of mean force (PMF) as a function of the centre-of-mass (COM) distance between one single peptide—which we gradually force to dissociate from the other segments—and the other three segments (simulation details are described in section SII of the Supplementary Information). For the scenario when LARKS are treated as fully disordered, we allow peptides to freely sample their conformational space (only fixing the position (in the appropriate direction) of the closest atom to the peptide COM of the structured four-peptide array; see SI for further details). In the second scenario, where we quantify the interactions among ordered LARKS, we constrain the peptides to retain their crystal-$\beta$-sheet structure\cite{8}.

Our simulations reveal that the interaction strength between disordered unconstrained peptides is sufficiently weak (i.e., < 0.5k_B T per residue) that, at room temperature, thermal fluctuations would frequently break and reform such inter-protein interactions, consistent with the formation of liquid-like condensates (Fig. 4, grey curve). More interestingly, when the peptides assemble into constrained inter-peptide cross-$\beta$-sheet structures, the strength of their interactions increases by almost an order of magnitude (i.e., to approximately 4k_B T per residue, red curve). To verify that our conclusions on the relative difference between disordered and structured binding are not model dependent, we also compute the PMF dissociation curve using the CHARMM36m force field\cite{117}. As shown in Fig. S9 of the SI, a ten fold difference between both peptide dissociation curves is also obtained in agreement with our calculations using the a99SB-disp force field\cite{116} (Fig. 4). We note, however, that the exact magnitude of this increase may be slightly overestimated by the constraints we have used to enforce the stability of the $\beta$-sheet structures (which likely contribute to increase the free energy of the $\beta$-sheet structure respect to the random coil, and therefore, the global minimum depth respect to the fully dissociated state). Nevertheless, these results, together with our coarse-grained simulations, suggest that an enhancement of inter-protein interactions may occur due to the formation of inter-peptide LARKS $\beta$-sheets, sufficient to sustain the formation of gels or aged solid-like aggregates. Importantly, the strength of structured LARKS–LARKS interactions remain sufficiently weak that they can still be considered thermolabile. Our results are also consistent with experiments reporting that LARKS-containing proteins form reversible hydrogels that can be easily dissolved with heat\cite{4,7,6,7}. A significant increase in the interaction strength after a disorder-to-order transition has been reported previously for the A$\beta$1–42 system\cite{17}. However, in the case of A$\beta$1–42, the observed increase was much larger, consistent with amyloid fibers being thermostable\cite{18,19}. Our previous coarse-grained simulations may reasonably describe the gradual rigidification that condensates can display due to the emergence of inter-protein $\beta$-sheet clusters (Fig. 4). Still, the free energy penalty associated to the structural transition, or more critically, the variation in binding strength after the cross-$\beta$-sheet formation is only approximated within our approach. Nevertheless, when we employ a time-dependent and spatially-dependent Hamiltonian coupled to a local order parameter\cite{20}, that enables variations in the binding strength among LARKS as a function of their local environment, we find consistent results of condensate maturation\cite{21} than those using a static model like the one in the present work (Figs. 3 and 4 of Ref.\cite{120}). Our simulations in Ref.\cite{120} reveal that a static model reasonably describes ageing due to strengthening of inter-protein interactions when the abundance of strongly-binding domains along the protein sequence is relatively low (Fig. 1A). Using a static model is necessary when dealing with extremely long system.
relaxation timescales and low protein self-diffusion as in the present work. A static model with low abundance of strongly-binding domains along the protein sequence can still adequately balance the β-sheet transition timescale (typically of the order of hundreds of nanoseconds125–127) and the protein self-diffusion timescale (of the order of hundreds of milliseconds128); thus, enabling the gradual rigidification of phase-separated condensates after protein aggregation23,34,49,65 as recently observed for different DNA- and RNA-binding proteins74,124,125.

Conclusions

In this work, we investigate the impact of enhanced inter-protein interactions in the modulation of the kinetic and thermodynamic properties of ageing biomolecular condensates. Our coarse-grained protein model shows that condensates remain liquid-like when proteins bind to one another weakly (i.e., < 1 kBT), but strengthening of inter-protein interactions (i.e., > 5 kBT per residue) gradually slows down the mobility of proteins over time, leading to progressive rigidification/maturation of the condensates23,49. We also observe that aged condensates exhibit a significant degree of hysteresis: once long-lived ordered—ordered interactions are established, amorphous condensates become heat resistant up to moderate temperatures close to the critical conditions for phase separation126. Consistently, our atomistic simulations, reveal that formation of inter-peptide β-sheets, such as those that may form within the LARKS regions of NUP-9874,125, and similarly in FUS, TDP-43 or hnRNPA1 among other proteins23,34,74,127, can increase the interaction strength between these segments significantly. Such strong binding variation may contribute to rationalise the physicochemical and molecular factors behind the intricate process of pathological maturation and formation of amorphous phase-separated condensates observed in LARKS-containing proteins such as FUS50, hnRNPA139, TDP-43124, or NUP-9874,109.

We also illustrate how the coupled effects of the decay in protein mobility, the timescale for the emergence of long-lived interactions, droplet coalescence times, and droplet size, crucially govern the shape and material properties of the condensates. When strong inter-protein binding occurs faster than droplet coalescence, the resulting condensates are non-spherical122,125. However, when the strengthening of protein interactions emerge after condensate formation (i.e., once a spherical droplet is already formed), the condensate only experiences a very slight deformation remaining mostly spherical. The time required for two separate tangant droplets to fuse and rearrange into a single spherical condensate depends on the initial size of the droplets that are attempting to fuse, and the strength of inter-protein interactions. In small condensates, where the rearrangement time is shorter than the timescale in which proteins lose their mobility due to clustering of structured motifs, condensates are mostly spherical but can eventually become kinetically arrested. In contrast, in larger droplets, where coalescence times are longer, the loss of protein mobility occurs faster than the time required for the condensate to rearrange, and therefore, protein aggregates become kinetically trapped in non-spherical or partially-fused states. Taken together, our results shed light on how local strengthening of inter-protein interactions—for instance due to formation of inter-protein β-sheets4,7,79,80, establishment of post-translational modifications41, or changes in salt conditions32—may impact the mesoscopic phase behaviour of biomolecular condensates, and suggest a mechanism for the emergence of aspherical droplets over time.

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