The role of liquid-liquid phase separation in aggregation of the TDP-43 low complexity domain

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ABSTRACT

Pathological aggregation of the transactive response DNA-binding protein of 43 kDa (TDP-43) is associated with several neurodegenerative disorders, including amyotrophic lateral sclerosis, frontotemporal dementia, chronic traumatic encephalopathy, and Alzheimer’s disease. TDP-43 aggregation appears to be largely driven by its low-complexity domain (LCD), which also has a high propensity to undergo liquid-liquid phase separation (LLPS). However, the mechanism of TDP-43 LCD pathological aggregation and, most importantly, the relationship between the aggregation process and LLPS remains largely unknown. Here, we show that amyloid formation by the LCD is controlled by electrostatic repulsion. We also demonstrate that the liquid droplet environment strongly accelerates LCD fibrillation and that its aggregation under LLPS conditions involves several distinct events, culminating in rapid assembly of fibrillar aggregates that emanate from within mature liquid droplets. These combined results strongly suggest that LLPS may play a major role in pathological TDP-43 aggregation, contributing to pathogenesis in neurodegenerative diseases.

Proteinaceous inclusions containing the transactive response DNA-binding protein of 43 kDa (TDP-43) are a pathological hallmark of sporadic ALS (1, 2) and are observed in numerous other neurodegenerative disorders associated with protein misfolding, including frontotemporal lobar degeneration with ubiquitin-positive inclusions (1, 3), Alzheimer’s disease (4–6), chronic traumatic encephalopathy (7), and cerebral age-related TDP-43 with sclerosis (8). Such a widespread observation of TDP-43 pathology underscores the need to gain molecular level insight into the aggregation pathway(s) of the protein, as this may be critical to understanding the underlying disease pathogenesis and to developing therapies.

TDP-43 consists of an N-terminal Dix-like domain (9) that mediates self-assembly (10), two RNA-recognition motifs, and an intrinsically disordered low complexity domain (LCD) at the C-terminus (11–13). An important feature of TDP-43 inclusions is the differential involvement of C-terminal fragments (CTFs), which are enriched in hippocampal preparations from patients (14). Studies have proposed a caspase-mediated cleavage event resulting in the robust production of CTFs of various sizes (25 kDa, 35 kDa) (1, 15, 16). Invariably, these CTFs contain the glycine-rich, disordered LCD (residues ~267-414) that appears to be of key importance to the intrinsic aggregation propensity of TDP-43 (12). Recent studies indicate, however, that the N-terminal domain is also important, playing a regulatory role in the formation of different types of aggregates (17). Even though the specific nature of TDP-43
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aggregates from patient histopathological samples is a topic for discussion (1, 18–20), the prion-like progressive behavior of TDP-43 proteinopathies (21–24) suggests that these aggregates have a seeding capacity characteristic of amyloids. Consistent with this notion, recent studies have revealed that the isolated LCD (13) and many of its fragments (25–31) readily form amyloid fibrils, while deletion of or within this region abrogates fibrillation (32–34). However, little is known about the mechanism of this amyloidogenic pathway.

The picture is further complicated by recent findings that, under certain experimental conditions, the TDP-43 LCD undergoes liquid-liquid phase separation (LLPS), whereby hydrophobic interactions and charge screening (35–38) facilitate assembly of reversible, liquid-like droplets. These dynamic species may subsequently convert to static assemblies (10, 35). For some other proteins that form liquid droplets, such as FUS (39) or hnRNPA1 (40, 41), aging of droplets was reported to eventually lead to the formation of amyloid fibrils. However, the impact of LLPS on the aggregation pathway of TDP-43 remains unexplored.

In this report, we describe biophysical studies on the relationship between LLPS of the TDP-43 LCD and its aggregation properties. The key finding of these studies is that LLPS conditions per se strongly promote TDP-43 amyloid fibril formation and that self-assembly of the protein is heavily regulated by electrostatic effects. These observations in vitro provide a foundation for understanding the role of LLPS in the formation in vivo of pathological TDP-43 aggregates within the context of neurodegenerative diseases.

Results

Conditions promoting liquid-liquid phase separation of the TDP-43 LCD

To systematically investigate the role of LLPS in pathological aggregation of the TDP-43 LCD, we began by characterizing its phase separation behavior under experimental conditions to be tested in this study. We utilized turbidity measurements (optical density at 600 nm) as a primary tool to delineate the liquid-liquid phase boundary of the TDP-43 LCD. Because both TDP-43 aggregation (12) and LLPS (35, 36) can result in observable turbidity, sole formation of liquid droplets was confirmed by optical microscopy. Similar to other reports (35, 36) that used buffer with pH around 6, we found that under these conditions the presence of salt induced LLPS for our protein. Significant droplet formation was observed by using Alexa488-labeled protein at ~150 mM NaCl, with a considerable increase in turbidity at higher salt concentrations (Fig. 1A, B).

Peculiarly, previous studies on the LLPS of the TDP-43 LCD focused almost exclusively on experimentation around pH 6–6.5 (35, 36). To explore in greater detail the effect of environmental conditions on the phase behavior of the protein, we performed additional experiments under different buffer conditions. We found that the propensity of the TDP-43 LCD to undergo LLPS is strongly pH dependent, requiring higher salt concentrations as pH is decreased (Fig. 1B). Importantly, at neutral pH, a small degree of LLPS was observed even in the absence of salt (Fig. 1A). Under all conditions, we consistently observed the promotion of LLPS by the presence of salt, with the threshold NaCl concentration for droplet formation increasing with a decreasing pH (Fig. 1B). Similarly, lower protein concentrations required higher salt concentrations to observe an increased turbidity (Fig. 1C).

Consistent with previous observations (35), we also found that temperature elevation to 37 °C at pH 6 resulted in no detectable turbidity of protein solution in the presence of up to 200 mM NaCl (Fig. 1D). This raised questions as to whether physiologically relevant conditions are indeed conducive to LLPS of TDP-43. To address this conundrum, we sought to emulate the crowded intracellular environment through the addition of the volume-excluding polymer, PEG (42). We found that the addition of PEG at typical concentrations used to mimic cellular crowding (43) resulted in LLPS at lower salt concentrations (25-50 mM) or even in the absence of salt at pH 6–conditions that are not conducive to LLPS when PEG is not present (Fig. 1E). Thus, crowding effects and charge screening appear to work in concert to facilitate LLPS.

With the observations that neutral pH, salt, and crowding agent all facilitate LLPS, we tested whether LLPS readily occurs under physiologically-relevant conditions (pH 7.3, 150 mM NaCl, 37 °C, 10% PEG). Under these conditions, we observed a small but significant change in turbidity between 2.5 μM protein concentrations (and becoming much larger at
higher concentrations) (Fig. 1F). Furthermore, spherical droplets were identifiable by microscopy, even though at the lowest protein concentrations these species were relatively small, with a diameter below 1 μm (Fig. 1G). Further confirming the physiological relevance of LLPS, we also found that droplets could be detected using low protein concentrations at pH 7.3, in the presence of 150 mM KCl, with or without 1 mM DTT (to mimic the environment of the nucleoplasm or cytoplasm, respectively) (Fig. 1H, I). Overall, these results demonstrate that physiologically relevant conditions are potentially conducive to TDP-43 LCD LLPS, providing a rationale for exploring the role of LLPS in pathological aggregation of the protein.

Salt promotes rapid self-association of TDP-43 LCD into oligomers

The above results demonstrated that LLPS of the TDP-43 LCD can be readily manipulated by experimental conditions and provided a foundation to explore biophysical and structural properties of the protein in relation to LLPS. Previous solution NMR studies (35, 36) of the LCD have revealed a partially α-helical region between residues ~320-340, which is proposed to mediate self-association of the protein into dimers, or possibly even larger multimers, as well as contribute to LLPS. However, the use of solution NMR to study protein liquid-liquid phase separation is not without limitations, as larger species may be undetectable (36). Here we used a complementary tool for structural study: site-directed spin labeling (SDSL) coupled with electron paramagnetic resonance (EPR) spectroscopy. The advantage of this approach is that EPR spectra are highly sensitive to local motions of a paramagnetic spin label attached to individual side chains, providing site-specific information about dynamics of all protein states within a sample (i.e., monomers, small multimers, and large aggregates), both in bulk solution as well as the phase-separated state. To facilitate these studies, we developed a library of spin-labeled variants of the TDP-43 LCD. Our library emphasized residues within the previously identified α-helical region (13, 30, 35), as these residues were the most likely candidates to be involved in potential conformational changes and intermolecular interfaces. All spin labeled variants described in our study were confirmed to not significantly alter the LLPS propensity of the LCD (Fig. S1). Because LLPS is strongly modulated by intermolecular interactions between the helices of LCD molecules (35), this suggests that Cys-variants used largely retained helical assembly capability; however, this was not directly ascertained.

In the absence of salt, EPR spectra of all variants tested (both at pH 6 and 4) depicted three relatively sharp lines, as illustrated in Fig. 2A for protein labeled at either position 280 or 333. Spectra of this type are characteristic of a highly dynamic (mobile) nitroxide label with a rotational correlation time on the order of $10^{-9}$ s (44). However, the amplitudes of spin-normalized spectra for labels at different positions show some variability, suggesting site-specific differences in mobility. Therefore, for more quantitative insight into residue mobility throughout the protein, we calculated for each spectrum the inverse of the width of the central line ($\Delta H_{0}^{-1}$), a frequently used EPR mobility parameter (45). Even though $\Delta H_{0}^{-1}$ values for all residues tested are in a range that is characteristic of high mobility (46), the motion appears somewhat more restricted (smaller $\Delta H_{0}^{-1}$) for residues within the ~323-337 region as compared to residues outside this region (Fig. 2B).

This is consistent with NMR data detecting partial α-helical structure at pH ~6 within this region (13, 30, 35, 36). Furthermore, the present EPR data, together with the similarity of CD spectra at pH 6 and 4 (Fig. S2A), suggest that the propensity for these residues to form an α-helix is preserved under acidic conditions.

Upon the addition of salt at pH 6 to induce LLPS, we immediately observed major changes in EPR spectra for some spin-labeled variants of TDP-43 LCD (all measurements were performed within 10 minutes of sample preparation). As exemplified in Fig. 2 C, D for the protein labeled at residue 327, these changes were characterized by diminished intensity of the spectral amplitude in spin normalized spectra (Fig. 2C) and the appearance of a second, broad spectral component that was most readily identifiable upon spectral scaling to the same amplitude of the central line (Fig. 2D) or after subtraction of the sharp component (Fig. 2D, inset). The appearance of the latter component clearly indicates the presence of a second population of protein molecules, in which the specific amino acid residue is more greatly immobilized, with rotational
Phase separation promotes aggregation of TDP-43 motions in the $10^{-4}$-$10^{-7}$ s timescale (44). The relative prevalence of this immobilized population can be assessed by calculating the ratio of peak intensities, $I_2/I_1$, for the broad and sharp spectral components as shown in Fig. 2D. Using this parameter, a residue-specific profile was developed for pH 6, 150 mM NaCl LLPS conditions and compared to that for the conditions with no LLPS (pH 6, 0 mM NaCl) (Fig. 2E). This profile indicates that the appearance of an immobilized component observed under LLPS conditions is largely limited to residues within the previously identified α-helical region, with some residues having a greater second component contribution than others [the non-zero intensity ratios in the absence of salt (grey bars, Fig. 2E) are due to a small contribution in this part of the spectrum from the mobile component].

In principle, the broad component observed in EPR spectra at pH 6 in the presence of salt (i.e., LLPS conditions) could potentially reflect folding of TDP-43 LCD monomers in such a way that specific residues become immobilized due to strong tertiary interactions. However, given previous NMR data (36), this scenario is unlikely. Other possibilities are that this immobilization results from rapid oligomerization of the protein, with the immobilized residues being involved in intermolecular interfaces, or that transient intermolecular interactions under LLPS conditions differentially alter the mobility of specific residues.

To further explore the latter two possibilities and test whether the presence of a residue-specific immobilized component at pH 6 in the presence of 150 mM NaCl is exclusively related to LLPS, we took advantage of the observation that the addition of moderate concentrations of salt (150 mM NaCl) at pH 4 results in oligomerization without inducing LLPS. As shown in Fig. 2F, this oligomerization reaction was clearly demonstrated by dynamic light scattering measurements (which could not be performed upon droplet formation at pH 6 due to severe light scattering). At pH 4 in the absence of NaCl, the TDP-43 LCD exists as a single species with a hydrodynamic radius ($r_H$) of ~2.1 nm and a predicted molecular weight of 16.8-18.6 kDa, strongly indicative of a monomeric nature. However, the addition of salt resulted in a clear bimodal distribution of species, with the smaller one likely corresponding to a higher-order species ($r_H = 2.7 \pm 0.7$ nm; predicted average molecular weight of 38.5 ± 22.8 kDa) and the larger one corresponding to much larger oligomers ($r_H = 13.8 \pm 3.5$ nm). This larger species was estimated to represent ~64% of the total protein mass, but was unlikely to be related to liquid droplet formation as no turbidity was observed and no droplet-like species were detected by microscopy at these conditions (Fig. 1A, B).

Importantly, the addition of 150 mM NaCl at pH 4 resulted in the appearance in EPR spectra of the second, immobilized component exclusively for residues within the ~325-335 region, with the peak intensity ratio profile qualitatively similar to that described above for LLPS conditions at pH 6 (Fig. 2E). In light of similar spectral changes being observed under both LLPS and non-LLPS (oligomer-forming) conditions, we reasoned that these findings would more likely reflect oligomer-specific interactions rather than more transient intermolecular interactions solely inherent to LLPS. However, due to large light scattering on liquid droplets, direct detection of oligomers under LLPS conditions was not possible. To ascertain whether oligomerization might also involve changes in secondary structure, CD spectra at pH 4 were recorded and found to be very similar in the presence and absence of salt (Fig. S2B). This further indicated that salt-induced changes in EPR spectra are largely (if not solely) due to protein self-association. Collectively, these data indicate that (i) salt induces rapid oligomerization of TDP-43 LCD in the absence of LLPS, with similar oligomerization possibly also occurring under the conditions of LLPS, and (ii) oligomerization interfaces appear to be limited to residues within the α-helical region.

**TDP-43 fibrillation in the absence of liquid-liquid phase separation**

Previous studies report that the TDP-43 LCD or its fragments can form amyloid fibrils (13, 25, 26, 28, 29). However, the mechanistic aspects of this reaction remain poorly understood. Limited studies addressing this issue using a longer C-terminal fragment construct were performed in a buffer containing 2.5 M urea, making interpretation of these data difficult (47). Even more important, no information is available regarding the relationship between fibril formation and LLPS of the TDP-43 LCD.

To build a foundation for understanding the role of LLPS in TDP-43 LCD amyloid formation,
we first studied fibrillation kinetics in the absence of LLPS. We found that incubation of the LCD at pH 6 in the absence of salt resulted in formation of fibrillar aggregates. Atomic force microscopy (AFM) imaging depicted these as non-clumped, well-dispersed fibers of ~5-10 nm in height (Fig. 3A). These aggregates exhibited a strong Thioflavin-T (ThT) fluorescence, suggesting a cross-β structure consistent with fiber diffraction reports (48) and allowing the monitoring of the kinetics of fibril formation via ThT fluorescence assay (Fig. 3B). These traces indicated classical fibrillation kinetics, with identifiable initial lag phase (corresponding to nucleus formation) followed by the fibril growth (elongation) phase (49). Consistent with observations for other amyloidogenic proteins (50, 51), the lag time duration was related to the concentration of protein, with higher concentrations promoting more rapid fibrillation (Fig. 3C).

Because salt appears to be critical to inducing initial rapid oligomerization of the TDP-43 LCD in the absence of LLPS (see Fig. 2), next we investigated the impact of increasing ionic strength (higher salt concentration) on fibrillation of the protein. We performed these studies at pH 4 because a broader range of salt concentrations could be tested without inducing LLPS. In the absence of salt, no ThT fluorescence could be detected during the time scale of the experiment (up to 30 hours). In contrast, we observed enhanced ThT fluorescence over time with diminishing lag times at increasing salt concentrations (Fig. 3D). Thus, the addition of salt under non-LLPS conditions appears to promote not only rapid formation of initial small oligomers but also the assembly of the TDP-43 LCD into amyloid fibrils.

**TDP-43 fibrillation under phase separation conditions**

Next, we turned our investigation to characterizing in greater detail the aggregation/fibrillation behavior of the protein under LLPS conditions. In addition to individual droplets, we observed relatively rapid formation (within ~15-30 min) of beaded structures that appeared to result from partial fusion events and deposited on the bottom of the microscope slide. This is exemplified in Fig. 4A for droplets formed at pH 7 and was also observed for LLPS at pH 4 and 6. Material properties of both droplet types were characterized by fluorescence recovery after photobleaching (FRAP), revealing a time-dependent decrease in maximal fluorescence recovery and rate of recovery (Fig. 4B). These results suggest a progression toward a gel-like, potentially aggregated state for both individual and beaded droplets.

In order to more closely examine the nature of aggregates formed within droplets, we employed AFM imaging. Freshly prepared droplets deposited and dried on a mica surface appeared as large spherical enclosures containing smaller particles, most likely representing patches of dried protein. AFM images of droplets deposited after different periods of aging show time-dependent morphological changes. Importantly, after several hours of incubation, these images clearly demonstrate fibrils emanating directly from the droplets (Fig. 4C). After longer incubation, there is a gradual progression to mostly (if not exclusively) fibrillar structures with varying degrees of clumping, which appears to be particularly severe at neutral pH (Fig. 4D). Thus, these data demonstrate that the formation of amyloid fibrils can occur within liquid droplets.

Next, we sought to explore the kinetics of TDP-43 LCD aggregation under LLPS conditions and to relate this to aggregation under non-LLPS conditions. ThT fluorescence traces at pH 6 revealed rapid polymerization under robust LLPS (200-300 mM NaCl) conditions, with lag phases markedly shorter compared to those observed in the absence of LLPS (Fig. 5A). The growth phases under LLPS conditions were rather complex, involving an initial rapid increase in fluorescence followed by a secondary, slower rate of increase toward steady state. These rapid phases, which were completed within ~3-3.5 h, corresponded to ~35-50% of maximal fluorescence intensity increase. By contrast, the reaction in the absence of LLPS (no NaCl) required ~10 h to reach the same ThT fluorescence response, further indicating a strong accelerating effect of LLPS on amyloid formation. It should be noted that Thioflavin-T is a rotor dye and its fluorescence properties can change as a function of solvent viscosity (52). Because LLPS occurs almost instantaneously with detectable turbidity, whereas fluorescence response indicative of aggregation occurs after a substantial lag phase (at least 1-2 hours), the present ThT data largely reflect the formation of amyloids as
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opposed to changes in viscosity related to LLPS. This is further confirmed by coincident AFM imaging depicting robust fibril growth.

Since salt accelerates fibrillation in the absence of LLPS at pH 4, we anticipated that salt would further accelerate aggregation under LLPS conditions. Though the fastest aggregation occurred under LLPS conditions (300 mM NaCl), to our surprise, the most significant changes in lag phase still occurred at relatively low salt concentrations. The magnitude of these changes strongly diminished at higher salt concentrations (that still do not result in LLPS), and this apparent “saturation” persisted into the two-phase regime (300 mM NaCl) (Fig. 5B). A similar trend was observed for lag phases at pH 6 (Fig. 5C). In this case, however, even lower salt concentrations were sufficient to promote rapid fibrillation. We reasoned that this effect of salt on fibrillation kinetics may be related to pH-dependent changes in charge repulsion among TDP-43 LCD molecules (53). At pH 4, 6, and 7, the predicted net charge of our protein is +13.2, +9.6, and +6.4, respectively (calculated from protcalc.sourceforge.net). At higher pH, protein-protein interactions would be enhanced by a decreased net charge repulsion, thereby enabling faster aggregation and a diminished responsivity to salt (54). Indeed, at pH 7, salt appeared to have minimal effect on lag phase (Fig. 5D). We subsequently explored the relationship between lag phase and LLPS (as assessed by initial sample turbidity) and found that, regardless of salt concentration or pH, all reactions under LLPS conditions were characterized by a very short lag phase of ~1-2 hours (Fig. 5E).

Overall, these results demonstrate that electrostatic effects regulate both LLPS and fibrillation kinetics and that conditions fostering robust LLPS coincide with those facilitating faster amyloidogenesis. To parse out the exact role of LLPS on TDP-43 LCD fibrillation while controlling for the effect of salt, we utilized additional LLPS regulators. First, we employed 5% 1,6-hexanediol, a hydrophobic reagent known to inhibit formation of liquid droplets (36). Turbidity measurements performed at pH 6 in the presence of 200 mM NaCl clearly indicate that this reagent indeed almost completely abrogates LLPS under these conditions (Fig. 6A). Importantly, the lag phase of the fibrillation reaction in the presence of 5% 1,6-hexanediol was markedly increased (Fig. 6B), indicating that the conditions of LLPS per se promote faster fibrillation of the TDP-43 LCD. As expected, no change in lag time was observed under control non-LLPS conditions (pH 4, 200 mM NaCl).

As opposed to using 1,6-hexanediol to disrupt LLPS, in a second experiment, we took advantage of the temperature sensitivity of TDP-43 LCD LLPS at pH 6. Consistent with data in Fig. 1, elevation of temperature to 37 °C at pH 6 in the presence of 200 mM NaCl resulted in essentially complete inhibition of the LLPS that is observed under identical buffer conditions at 25 °C (Fig. 6C). Again, this abrogation of LLPS coincided with a substantial increase in the duration of the lag phase of fibrillation reaction at pH 6 (Fig. 6D). In contrast, the lag phase under control non-LLPS conditions (pH 4, 200 mM NaCl) was actually slightly shorter at 37 °C as compared to that at 25 °C, an expected outcome for non-phase-separating amyloidogenic proteins (55). Altogether, these data consistently point to a direct role of LLPS as a mechanism for promoting TDP-43 LCD polymerization into amyloid fibrils.

**Discussion**

A recent series of exciting studies has demonstrated that a number of aggregation-prone proteins involved in neurodegenerative diseases can undergo LLPS (56–58). For some (e.g., FUS, hnRNPA1), the droplet environment is quite conducive to amyloid formation (39–41). Among these LLPS-prone proteins, TDP-43 is of particular interest because its aggregation is associated with a host of neurodegenerative disorders, including ALS, frontotemporal dementia, chronic traumatic encephalopathy, and Alzheimer’s disease (1, 3–5, 7). In the absence of LLPS, the TDP-43 LCD has recently been demonstrated to form amyloid fibrils (13); however, the mechanism of this process is poorly understood. An even more critical knowledge gap involves the connection between LLPS and the aggregation pathway of TDP-43. Insight into this relationship is of potentially crucial importance, as LLPS may play a regulatory role in the pathogenesis of neurodegenerative disorders. In the present study, we have expanded the current understanding of the conditions and interactions that regulate LLPS of the TDP-43 LCD in vitro and, on this background, explored the impact of LLPS on the aggregation behavior of the protein.
Initial work on the LLPS of the TDP-43 LCD focused on experiments around pH 6 and revealed that the addition of salt promotes liquid droplet formation (35–37). These findings emphasized the importance of unfavorable electrostatic repulsion that decreases the propensity for phase separation and of favorable hydrophobic interactions that are essential for droplet formation. Here, we show that pH and molecular crowding also play a regulatory role in LLPS, with the requirement for salt diminishing as pH approaches neutral values. These combined effects favor LLPS under physiologically-relevant buffer conditions and protein concentrations. Importantly, our data also reveal that electrostatic repulsion is a pervasive regulatory element not only of phase separation but also of protein oligomerization and, eventually, the formation of amyloid fibrils. A previous study proposed that neutral pH is required for fibrillation of TDP-43 LCD (13). However, no kinetic curves were presented and, in our experience with using a similar purification protocol, the 1 mM phosphate solution used in those biophysical experiments may not have sufficient capacity to buffer trifluoroacetic acid salts present upon reconstitution of HPLC-purified protein. This apparent pH dependence was suggested to result from ostensible intramolecular hydrogen bond networks (between backbone amide protons and side chain oxygen atoms) at acidic pH that would break down at more neutral pH, leading to faster fibrillation (13). However, as noted in a commentary on this study (53), the presence (and disruption by changes in pH) of such a network in the LCD is highly unlikely. Our present data that demonstrate a strong accelerating effect of salt on fibrillation kinetics provide direct experimental support to an alternative explanation (53), which argues that electrostatic repulsion (which diminishes at neutral pH or in the presence of salt) governs the pH dependence of TDP-43 LCD fibrillation.

Along the same line, we find that the same electrostatic repulsion that regulates LLPS and fibrillation of TDP-43 LCD is intrinsically linked to rapid formation of early (non-ThT-reactive) oligomers in the presence of salt. Oligomerization appears to be stabilized by intermolecular interactions that involve the same α-helical region that has been proposed to be important for LLPS (35, 36). The formation of multimers has been previously suggested based on weakening NMR signal intensities (13, 35, 36). Our present study provides a more direct evidence for this reaction and reveals that it is regulated by electrostatic effects. Furthermore, our spin labeling/EPR experiments suggest that rapid oligomerization of TDP-43 LCD also occurs within the context of liquid droplets. Since oligomeric species would be expected to diffuse relatively slowly, our FRAP data indicate that droplets remain dynamic beyond the time period required for rapid protein oligomerization. This suggests that these initial oligomers might also have a dynamic character, allowing for rapid exchange of the monomers.

The key finding of our present investigation is that LLPS is highly conducive to the formation of amyloid fibrils by the TDP-43 LCD. The aggregative process under LLPS conditions appears to include several distinct stages, whereby the initial events are characterized by relatively rapid self-association of the LCD into oligomeric species and subsequent changes in material properties within liquid droplets (as detected by loss of dynamicity via FRAP). These events are followed by the assembly of Thioflavin-T reactive fibrillar aggregates. Though it was not a primary focus of the seminal work (35) by Conicella and colleagues, who first characterized the LLPS of the TDP-43 LCD, ThT-reactive species were not observed to form within mature droplets. This suggested that aggregation under LLPS conditions may follow a non-amyloidogenic pathway. However, our present data clearly demonstrate that this is not the case. In fact, LLPS appears to promote the amyloidogenic pathway, as indicated by substantially faster overall fibrillation kinetics (and remarkably shorter lag times) as compared to those in the absence of LLPS. AFM imaging clearly depicts fibrils emanating from within droplets (Fig. 4C). The notion that LLPS facilitates TDP-43 LCD amyloidogenesis is further supported by experiments disrupting droplet formation (while controlling for changes in electrostatic repulsion) via elevated temperature or the addition of 1,6-hexanediol, both of which result in slower fibrillation. In contrast, in the absence of LLPS, higher temperatures instead appear to facilitate faster fibrillation, similar to the observations for other amyloidogenic proteins in bulk solution (55). Thus, LLPS is playing a regulatory role in pathological aggregation of TDP-43 LCD.

In contrast to fibrillation reactions in the
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absence of LLPS that depict classical polymerization kinetics, traces under LLPS conditions are far more complex. These are characterized by a lag phase that is followed by at least two distinct stages of ThT fluorescence increase associated with fibrillar growth (Fig. 5A). This is not surprising, given that in a two-phase system the protein can aggregate both within liquid droplets as well as in the surrounding bulk solution. The first of these growth stages is very rapid, likely representing fibrillation occurring within droplets where the protein is highly concentrated. The origin and interpretation of the second, much slower growth phase is more complicated. This stage may represent contributions from both a spontaneous fibrillation reaction in the bulk solution as well as a reaction in the bulk solution that is seeded with fibrillar aggregates (that rapidly formed within the droplets). However, the efficiency of this seeding might not be very high since these droplet-associated fibrils appear to be highly clumped.

Our current findings regarding the impact of LLPS on the aggregation properties of TDP-43 are limited to experiments in vitro with a purified LCD. As such, these findings do not account for the potential role of the TDP-43 N-terminal domain, which may alter the aggregation landscape by modulating phase separation and the formation of pathogenic species (10, 17, 59, 60). It is likely that the reported recruitment of TDP-43 to cytoplasmic stress granules in vivo (61, 62) may involve similar self-association of TDP-43 molecules, thus triggering robust LLPS-mediated fibrillation. Indeed, the notion that LLPS may mediate formation of pathological aggregates of TDP-43 in neurodegenerative diseases is supported by recent observations that TDP-43 aggregates appear to localize to stress granules in Drosophila and mouse models of traumatic brain injury (63, 64). Consistent with these findings, our data suggest that LLPS plays a direct role in facilitating aggregation of the TDP-43 LCD, likely by providing an environment of increased local concentration of the protein. However, recent evidence from a cell culture model suggests that these aggregates eventually dissociate from stress granules (65). Detailed elucidation of the mechanisms of formation of these aggregates is of great importance not only for understanding the pathogenic process, but also for developing therapeutic approaches. On this front, recent structural investigations of TDP-43 LCD peptides have revealed a labile, intermediary type of interaction (referred to as low-complexity aromatic-rich kinked segments, or LARKS) that may subsequently become irreversible (48). These types of interactions could be involved in the observed early changes in material properties of droplets during the lag phase of amyloid aggregation and would therefore be optimal for therapeutic intervention. Indeed, a chemical chaperone has recently been shown to promote LLPS of TDP-43 while abrogating amyloid formation, although aggregation within the context of liquid droplets was not directly examined in this study (66). Even though further studies are needed to fully understand the link between LLPS and TDP-43 aggregation in vivo, our findings ultimately posit that LLPS is a viable, biologically-relevant mechanism under which the formation of amyloid aggregates by TDP-43 may occur and contribute to the pathogenicity of neurodegenerative disorders.

Experimental Procedures

Expression, Purification, and Preparation of Wild-type and Cys Variants

The LCD sequence was subcloned into a pRSET-B vector from the full length TDP-43, originally obtained as a gift from Aaron Gitler (Addgene plasmid #27462). The construct was designed to encode the LCD protein (residues 267-414) with an N-terminal 6x His-tag and thrombin cleavage site (MRGSHHHHHHLVPRGS). His-tag removal consistently resulted in low yields of purified protein, possibly due to the high aggregation propensity at conditions necessary for efficacious thrombin activity. Though the presence of a histidine (with a pKa of ~6) likely has an influence on the pH-dependence of the LCD (which does not naturally contain histidine), use of a His-tagged protein is in line with almost all previously published studies exploring the LLPS or aggregation properties of purified TDP-43 LCD (13, 36, 67). A notable exception is the study by Conicella et al., in which the authors removed the His-tag, but which still included artificial GH residues at the N-terminus (35). However, in our experience, the recovery of the cleaved protein used in our present study (which would not contain the extra His residue) was very low. Therefore, we used His-tagged protein, which could then be purified in...
quantities sufficient for systematic biophysical studies.

Protein was expressed in BL21 (DE3) E. coli overnight and bacteria were lysed by sonication in Buffer A (20 mM Tris-HCl buffer, pH 8, containing 8 M urea, 500 mM NaCl, and 25 mM imidazole). Protein was purified on Ni-charged nitrilotriacetic acid (NTA) column, using 4-5 column volume washes with Buffer A, and subsequent elution in Buffer B (20 mM Tris-HCl buffer, pH 8, containing 8 M urea, 200 mM NaCl, and 250 mM imidazole). This was followed by a final purification step by HPLC using a C4 column acetonitrile gradient in water containing 0.05% trifluoroacetic acid. Purified protein (better than 95% purity as assessed by gel electrophoresis and mass spectrometry) was flash frozen and lyophilized.

Single-Cys variants were generated by site-directed mutagenesis, expressed and purified by NiNTA as described above but in the additional presence of 1 mM TCEP. Labeling of Cys variants was performed for 1-2 hr in Buffer B (+ 1 mM TCEP) with >15-fold molar ratio of (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrroline-3-methyl) Methanethiosulfonate (MTSL, Toronto Research Chemicals) to protein (~10 mg/mL LCD protein). Labeling efficiency was confirmed by mass spectrometry. Free label was removed using 7 kDa cut-off Zeba spin desalting columns equilibrated with Buffer C (20 mM Tris buffer, pH 8, containing 8 M urea, and 150 mM NaCl) to maximize protein recovery. The final purification step involved HPLC as described above.

Alexa488 fluorescently-labeled protein was prepared by attaching AlexaFluor 488 C5 Maleimide (ThermoFisher) to the protein variant with Cys at position 275. After labeling according to manufacturer’s instruction (in Buffer B containing 1 mM TCEP), excess label was removed by spin desalting column equilibrated with Buffer C.

Immediately before experiments, lyophilized protein was dissolved in Milli-Q H2O (~200-400 μL) and passed through a 0.5 mL 100 kDa Amicon Ultra centrifugal filter unit. Filtered protein was confirmed to be monomeric by dynamic light scattering and its concentration was assessed by absorbance at 280 nm using the extinction coefficient of 17990. The protein was then buffered to the appropriate pH using 10 mM sodium acetate (pH 4) or 10 mM potassium phosphate (pH 6 and 7).

**Fluorescence Microscopy and FRAP Measurements**

Bright field and fluorescence microscopy imaging studies for Fig.1 only were acquired using a Keyence BZ-X700 microscope with 100x/1.45 oil immersion lens. The microscope uses a metal halide lamp, GFP filter cube (excitation: 470 nm; DM 495), monochrome CCD, and Nikon CF160 series infinite optical system. Fluorescence microscopy images were taken using a 1:200 molar ratio of Alexa488-labeled to unlabeled protein. Processing of bright field images involved a haze reduction filter to highlight identifiable, small species. Processing of fluorescence images involved black balance correction only.

Fluorescence recovery after photobleaching (FRAP) was performed using a Leica SP8 confocal microscope with 2.4 mW laser intensity for bleaching, 63x/1.4 oil immersion objective, and PMT detector. Each FRAP experiment involved 5 pre-bleach frames, followed by 10 bleach frames, and recovery was monitored over 300 s (1 frame/5 s). Because multiple droplets could be observed in each frame, 3 droplets were bleached at a time for each experiment. FRAP analysis involved assessment of mean fluorescence intensity from each bleached region of interest (ROI) as well as non-bleached ROIs to correct for microscope drift. Individual FRAP traces were normalized to maximal pre-bleach and minimal post-bleach intensities.

**Turbidity and Thioflavin-T Fluorescence Assays**

Turbidity (optical density at 600 nm) measurements were performed using an M1000 Tecan plate reader. The reported A600 values reflect initial turbidity at 25 °C or once the plate reached 37 °C. Kinetic traces for fibrillation were acquired by measuring ThT fluorescence (dye concentration of 15 μM) on the M1000 Tecan plate reader with excitation and emission wavelengths of 440 and 485 nm, respectively. Measurements were taken every 10 min with no additional agitation. Lag phases for aggregation reactions were quantified as the time point of intersection between a line drawn through the elongation phase data points and a line drawn through the lag phase data points (55).

**EPR Spectroscopy**
EPR spectra were obtained at ambient temperature on a Bruker EMX spectrometer outfitted with a high-sensitivity resonator. The measurements were performed using field modulation of 2 G and a scan width of 200 G.

Circular Dichroism Spectroscopy
Spectra were recorded in a 1 mm path length quartz cuvette at 25 °C using an AVIV Model 215 spectrometer.

Dynamic Light Scattering
Measurements were performed in a disposable cuvette using a DynaPro NanoStar dynamic light scattering instrument (Wyatt Technology) at 25 °C (25 acquisitions per measurement with 5 s averaging time). Data were analyzed using built-in Dynamics software.

Atomic Force Microscopy Imaging
Samples were deposited on freshly-cleaved mica substrate, left at ambient temperature for ~3-5 minutes, then washed four times with fresh Milli-Q H2O and dried under N2. The images were obtained using scan assist mode and a silicon probe (spring constant: 40 N/m) on a Bruker multimode atomic force microscope equipped with Nanoscope V controller, as described previously (68). Image processing was performed using Nanoscope Analysis software.
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The abbreviations used are: TDP-43, transactive response DNA-binding protein of 43 kDa; LCD, low complexity domain; LLPS, liquid-liquid phase separation; SDSL, site-directed spin labeling; EPR, electron paramagnetic resonance; DLS, dynamic light scattering; OD, optical density; PEG, polyethylene glycol; FRAP, fluorescence recovery after photobleaching; ROI, region of interest.
Phase separation promotes aggregation of TDP-43

Figure 1. Liquid-liquid phase separation of the TDP-43 LCD. (A) Representative fluorescence microscopy images of 20 μM LCD at pH 4, 6, and 7 with varying salt concentrations at 25 °C using a 200:1 ratio of unlabeled to Alexa488-labeled protein. (B) LLPS of TDP-43 LCD as monitored by turbidity (OD @ 600 nm) at varying pH and salt concentrations, 25 °C. Protein concentration: 20 μM. (C) LLPS as monitored by turbidity at decreasing protein concentrations in the presence of varying amounts of salt. Protein concentration: 20 μM. Buffer conditions: 10 mM potassium phosphate (KP) pH 6. (D) Turbidity as a measure of LLPS at 25 °C and 37 °C with increasing salt concentrations. Protein concentration: 20 μM; buffer conditions: 10 mM KP pH 6. (E) Turbidity measured with increasing concentration of PEG-10,000 and various concentrations of NaCl. Protein concentration: 20 μM; buffer conditions: 10 mM KP pH 6, 25 °C. (F) LLPS as monitored by turbidity as a function of protein concentration. Buffer conditions: 10 mM KP pH 7.3, 150 mM NaCl, 10% PEG-10,000, 37 °C. (G) Representative bright field images corresponding to conditions described in panel F; scale bar: 5 μm. (H) LLPS as monitored by turbidity as a function of protein concentration. Buffer conditions: 10 mM KP pH 7.3, 150 mM KCl, 10% PEG-10,000, 37 °C, with and without 1 mM DTT. (I) Representative bright field images corresponding to conditions in panel H; scale bar: 5 μm.
Figure 2. Rapid oligomerization of TDP-43 in the context of LLPS. (A) Representative EPR spectra of TDP-43 LCD (20 μM) spin-labeled at position 280 (black) and 333 (red). Spectra were recorded at pH 4 in the absence of NaCl. (B) The mobility parameter (inverse of the central line width, ΔH₀⁻¹) for residues probed by SDSL at pH 4 and 6, both in the absence of NaCl. (C) Spin-normalized EPR spectra for TDP-43 LCD labeled at residue 327 at 0 (black) and 150 mM NaCl (blue); protein concentration: 20 μM. (D) Scaling of spectra shown in panel C to the same intensity of the central line clearly reveals the appearance in the presence of 150 mM NaCl of a second broad component with intensity, I₂, that is distinct from the first, sharp component with intensity, I₁. (Inset) The second component can be even better visualized via spectral subtraction of the first component. (E) Intensity ratios (I₂/I₁) of the broad-to-sharp components (as defined in panel D) for residues probed by SDSL in the absence (gray) and presence of 150 mM NaCl (blue) at pH 6 (left; LLPS was observed in the presence of salt) and pH 4 (right; no LLPS under either condition). (F) Size distribution of TDP-43 LCD at pH 4 with and without 150 mM NaCl. Protein concentrations: 100 μM; 25 °C.
Figure 3. TDP-43 LCD fibrillation in the absence of LLPS. (A) Atomic force microscopy image of fibrillar aggregates formed from 20 μM protein in the absence of NaCl at 25 °C. (B) Representative Thioflavin-T (ThT) fluorescence intensity trace for 5 μM TDP-43 LCD incubated without NaCl at pH 6, 25 °C (no LLPS). (C) Lag times derived from ThT fluorescence traces for LCD incubated in the absence of salt at pH 6, 25 °C at varying protein concentrations. Error bars represent standard deviation. (D) Representative ThT fluorescence kinetic traces for 20 μM LCD incubated at pH 4 with various salt concentrations, 25 °C (each trace shown represents an average of three experiments).
Figure 4. Formation of TDP-43 amyloid fibrils within liquid droplets. (A) Representative fluorescence microscopy images for FRAP experiments on individual droplets (left) and beaded droplets (right) prepared from 50 μM LCD and 100 nM Alexa488-labelled LCD in pH 7 buffer with 300 mM NaCl. Scale bar: 5 μm. (B) Fluorescence recovery traces after photobleaching for experiments illustrated in panel A. Each trace represents an average of at least three droplets and error bars represent standard deviation. (C) Atomic force microscopy images of TDP-43 LCD droplets deposited and dried on mica at various time points during incubation at 25 °C under LLPS condition in a buffer containing 300 mM NaCl at pH 4 (upper images) or pH 6 (lower images). Protein concentration: 20 μM. White scale bars correspond to 400 nm. (D) Representative atomic force microscopy images of fibrils after prolonged (6 days) incubation of TDP-43 LCD under LLPS conditions at pH 4, 6, or 7 in the presence of 300 mM NaCl. Scale bars: 400 nm.
Figure 5. LLPS strongly accelerates fibrillation of TDP-43 LCD. (A) ThT fluorescence traces for 20 μM LCD incubated at 25 °C in pH 6 buffer with various concentrations of NaCl. (B, C, and D) Lag phase time from ThT kinetic traces at pH 4 (B), pH 6 (C), and pH 7 (D) at various concentrations of NaCl. Protein concentration: 20 μM. (E) Lag phase time of fibrillation as a function of initial turbidity depicting data accumulated in this study at conditions of different pH and salt concentrations. Data in (B-E) represent averages from experiments using at least three separately prepared batches of protein. Protein concentration: 20 μM. Error bars represent standard deviation.
Figure 6. LLPS intrinsically promotes TDP-43 LCD fibrillation independent of electrostatic effects. (A) Turbidity and (B) fibrillation lag phase time for 20 μM protein at 25 °C in the presence or absence of 5% 1,6-hexanediol. Buffer conditions: pH 4, 200 mM NaCl (control, no LLPS with and without 5% 1,6-hexanediol) or pH 6, 200 mM NaCl (LLPS in absence of 5% 1,6-hexanediol only). (C) Turbidity and (D) fibrillation lag phase time for 20 μM protein at either 25 °C or 37 °C. Buffer conditions: pH 4, 200 mM NaCl (control, no LLPS at both temperatures) or pH 6, 200 mM NaCl (LLPS at 25 °C only) (no 1,6-hexanediol was used). Error bars represent standard deviation.
The role of liquid-liquid phase separation in aggregation of the TDP-43 low complexity domain

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