Measles virus nucleo- and phosphoproteins form liquid-like phase-separated compartments that promote nucleocapsid assembly

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Many viruses are known to form cellular compartments, also called viral factories. Paramyxoviruses, including measles virus, colocalize their proteomic and genomic material in puncta in infected cells. We demonstrate that purified nucleoproteins (N) and phosphoproteins (P) of measles virus form liquid-like membraneless organelles upon mixing in vitro. We identify weak interactions involving intrinsically disordered domains of N and P that are implicated in this process, one of which is essential for phase separation. Fluorescence allows us to follow the modulation of the dynamics of N and P upon droplet formation, while NMR is used to investigate the thermodynamics of this process. RNA colocalizes to droplets, where it triggers assembly of N protomers into nucleocapsid-like particles that encapsidate the RNA. The rate of encapsidation within droplets is enhanced compared to the dilute phase, revealing one of the roles of liquid-liquid phase separation in measles virus replication.

INTRODUCTION

Measles virus (MeV) is a highly contagious virus that is responsible for more than 100,000 deaths every year. No treatment currently exists for MeV infection, and the impact of MeV throughout the world has recently been amplified by weakening levels of vaccine coverage (1). MeV is a nonsegmented, single-stranded negative-sense RNA virus (Mononegavirales) of the family Paramyxoviridae, whose members carry their own replication machinery comprising nucleoprotein (N), polymerase (L), and phosphoprotein (P) components. N binds to the viral genome, assembling into helical nucleocapsids, while P chaperones N before encapsidation and is thought to bring N and L into close spatial proximity during replication and transcription (2, 3). The replication process represents a promising but hitherto relatively unexplored therapeutic target.

N and P both comprise domains exhibiting high levels of intrinsic disorder, with respectively 30 and 75% of their primary sequences lacking tertiary structure. N has a 125–amino acid disordered C-terminal domain (N_TAIL) (4) and a flexible 30–amino acid N-terminal arm (N_ARM), flanking the RNA binding domain N_CORE, while P comprises disordered domains of 304 amino acids (P_TAIL) (5) and 80 amino acids (P_LOOP), on either side of a coiled-coil tetrameric domain (6), and is capped by a C-terminal three-helix bundle (XD) (Fig. 1A) (7). N and P exhibit numerous interactions that are essential during different stages of the viral cycle: Two distant linear motifs in P_TAIL, P_1–50, and P_168–198, participate in chaperoning the monomeric form of N (5, 8), preventing binding to cellular RNA. In an N^2P heterodimer, P_1–50 binds to the same surface of N as N_ARM of the succeeding protomer in assembled nucleocapsids (9), so that the N^2P complex efficiently inhibits assembly. XD is known to bind to N_TAIL (10, 11), an interaction thought to be important for colocalizing N and L during the replication process and was recently shown to interact with L (12). The process of nucleocapsid assembly on viral RNA sequences was recently studied in real time, using nuclear magnetic resonance (NMR) and fluorescence (13), highlighting the role of N_ARM and P_1–50, and leading to high-resolution structures revealing N-RNA interactions in assembled nucleocapsids (14).

In recent years, liquid-liquid phase separation (LLPS), driven by weak interactions often involving intrinsically disordered proteins or RNA, has been shown to offer a highly efficient means to spatially and temporally control a remarkable array of cellular processes (15). The physicochemical properties of these microenvironments are thought to provide optimal conditions to enhance specific molecular interactions within the condensate, while maintaining immiscibility with respect to other excluded molecules (16–18). A number of viruses have been shown to form cytoplasmic compartments in infected cells, known as “viral factories,” that have been associated with the viral replication process (19, 20). Colocalization of N and P in vivo is a characteristic feature of mononegaviruses such as rabies (21), vesicular stomatitis virus (22), respiratory syncytial virus (23), human parainfluenza types 3 (24) and 5 (25), human metapneumovirus (26), Nipah (27), and MeV (28, 29). Inclusion bodies from MeV have been shown to colocalize host proteins such as WDR5 (WD repeat-containing protein 5) (28) and other viral proteins [the polymerase L as well as C that is involved in controlling host immune response (30)]. Recent confirmation of the liquid-like nature of Negri bodies formed in rabies (31) and colocalization of N and P in infection-induced cytoplasmic inclusion bodies in MeV (32) suggests that LLPS represents a widespread mechanism exploited by mononegaviruses during infection.

Here, we demonstrate that mixing MeV N and P proteins in vitro triggers LLPS, allowing us to characterize the physical nature of the droplets, including the modulation of the dynamic behavior of N and P upon droplet formation, the molecular basis of the stabilizing interactions, and the thermodynamics of droplet formation. We are able to show that RNA molecules preferentially localize to the droplets and, once within the droplets, trigger assembly of nucleocapsid-like particles. The process of nucleocapsid assembly in LLPS is observed in real time using NMR spectroscopy. The rate of assembly is significantly enhanced compared to non–phase-separating conditions.

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providing clear evidence that LLPS has the ability to enhance viral function and supporting previous suggestions that LLPS is exploited by negative-strand RNA viruses to create viral replication factories.

RESULTS
MeV N and P proteins phase separate upon mixing in vitro
Recombinant full-length P (P_{1-507}) and an RNA-free N^{59}P complex (P_{50}N_{525}) were separately expressed and mixed together and observed to form micron-sized spherical structures (Fig. 1B). Phase separation of P_{1-507} and full-length N was also demonstrated by coexpressing both proteins using separate plasmids in *Escherichia coli*. The full N^{59}P complex was purified in a high-salt buffer to prevent phase separation (vide infra) and diluted into low salt, resulting in phase separation as observed from differential interference contrast (DIC) microscopy (Fig. 1B).

Identification of N and P domains that are required for phase separation
To identify which domains of MeV N and P are essential for phase separation, we tested a panel of different N and P constructs for their ability to trigger phase separation (Fig. 1, A to C, and fig. S1). Several mixtures formed condensed phases: P_{50}N_{525} + P_{1-507}, P_{50}N_{525} + P_{304-507}, and P_{300}N_{525} + P_{304-507} (Fig. 1B). P_{50}N_{525} + P_{304-507} was thus identified as the minimal phase-separating system, forming spherical bi-molecular condensates as measured by fluorescence microscopy (Fig. 1B) and showing increased turbidity (Fig. 1D). Further investigations of MeV droplets were performed using this minimal system (P_{50}N_{525} and P_{304-507}) unless otherwise stated. P_{50}N_{525} and P_{304-507} colocalize in droplets (Fig. 1E), and their phase transition was shown to be salt dependent: Beyond 400 mM, fluorescent speckles are no longer observed (fig. S2).

The phase diagram of LLPS was established on the basis of turbidity (Fig. 2A), identifying threshold concentrations of 10 µM for both proteins, although fluorescence microscopy and negative-stain electron microscopy reveal phase separation at lower protein concentrations. Fluorescence microscopy indicates that proteins are homogeneously distributed within droplets (fig. S3).

The liquid-like behavior of droplets was confirmed by DIC microscopy via observation of fusion events with relaxation into spherical droplets (Fig. 2B and movie S1) and further demonstrated...
by fluorescence recovery after photobleaching (FRAP) of fluoresceinlabeled P50N525, showing recovery within seconds (Fig. 2, C and D). FRAP of P protein is measurably slower (Fig. 2E), suggesting that the branched multimeric P acts as a dynamic scaffold for the liquid droplets (33). Rotational diffusion of proteins within droplets and in the surrounding phase was measured using time-resolved fluorescence anisotropy. The rotational correlation time of P50N525 in the presence of P304–507 was significantly slowed down compared to P LOOP, demonstrating that P LOOP remains disordered in the context of the construct that links the tetramerization domain and XD and that is required for phase separation (see fig. S6).

The N TAIL:P XD interaction is essential for droplet formation

The C-terminal domains of N and P are known to interact via residues 487 to 501 of N TAIL, which populates a conformational equilibrium of partially folded helices in solution (34) and the XD domain of P. The affinity of the MeV N TAIL and P XD (Phosphoprotein XD domain) interaction (7, 11) under our experimental conditions is approximately 5 μM (Fig. 3, A to C). This affinity was not measurably dependent on salt concentration. The sole intermolecular hydrogen bonding interaction observed in a chimeric crystal structure of N486–505:P XD involves S491 of N TAIL interacting with K489 and D493 of P XD (7). Mutation of N (S491) has been shown to abrogate the N TAIL:P XD interaction—this is also the case under our experimental conditions (Fig. 3D)—and to significantly decrease viral transcription in vivo (11). The critical nature of this interaction for LLPS was significantly populated secondary structure with the exception of two regions with 20 and 10% α-helical propensity around residues 406 and 433, respectively. 15N relaxation data also show that this domain is highly dynamic (fig. S5), although relaxation rates around regions 410 and 433 increased in constructs containing the tetrameric or XD domains (P377–507 and P304–507), probably due to slower rotational tumbling. Carbon chemical shifts of P304–507 did not change significantly in comparison with P LOOP, demonstrating that P LOOP remains disordered in the context of the construct that links the tetramerization domain and XD and that is required for phase separation (see fig. S6).

P LOOP is intrinsically disordered

The conformational behavior of the region between the tetramerization domain and XD, P377–457 (P LOOP), was analyzed using NMR spectroscopy. 13C, 15N, and 1H chemical shifts were combined with statistical coil sampling and ensemble selection approach ASTEROIDS (fig. S4), revealing that this domain is essentially devoid
demonstrated via the S491L mutation, which resulted in suppression of phase separation.

The P LOOP-N interaction modulates the dynamics of MeV droplets

An additional interaction between P LOOP and N was identified using NMR spectroscopy (Fig. 4, A and B), suggesting that the role of this domain, which is present in all constructs involved in phase separation, involves a direct interaction with N rather than acting as a simple spacer (35). Interactions centered on residues 409 VLK 411 and 433 LLK 435 exhibit distinct affinities \([\text{estimated dissociation constant (KD)} = 264 \text{ and } 606 \mu M, \text{respectively}]\). Mutation of 409 VLK 411 to AAA inhibited this interaction (Fig. 4C) with N and modulated macroscale dynamic properties of droplets, as measured from FRAP, but did not completely inhibit LLPS (Fig. 4D). Both 409 VLK 411 and 433 LLK 435 exhibit high sequence conservation over MeV phosphoproteins (fig. S7).

Investigating the thermodynamics of droplet formation using NMR

To study interactions between proteins under phase-separating conditions, we compared \(^{15}\text{N} \) heteronuclear single-quantum coherence (HSQC) spectra of P XD and N 460–525 separately and in different stoichiometric mixtures. Residues from 377 to 507 were observable in the PXD spectrum, while N TAIL and flexible residues corresponding to N ARM and P 1–50 were observable in P 50 N 525 (Fig. 5). Overall peak intensity was initially observed to decrease for both N and P with increasing stoichiometric ratio (N/P), with no noticeable chemical shift differences. Similar observations were recently made in mixtures of two interacting translational regulators (36). Apparent rotational and transverse diffusion rates were similar to those measured for free proteins (fig. S8). Rotational correlation times would be expected to increase significantly within droplets due to increased viscosity (37). This result therefore suggests that observable signal derives from N and P in the dilute phase.

Decrease in resonance intensity of N and P is accompanied by broadening of resonances from P XD and N 460–525, due to the expected interactions between P and N. Modulated macroscopic properties of droplets, as measured from FRAP, but did not completely inhibit LLPS (Fig. 4D). Both 409 VLK 411 and 433 LLK 435 exhibit high sequence conservation over MeV phosphoproteins (fig. S7).


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Decrease in resonance intensity of N and P is accompanied by broadening of resonances from P XD and N 460–525, due to the expected interactions between P and N. Increasing the N:P ratio further results in a minimum of total peak intensity at a ratio of approximately 0.15, followed by continual increase (Fig. 5). To follow the apparent thermodynamics of droplet formation, intensities of 23 (N) and 9 (P) nonoverlapping peaks corresponding to residues that are not affected by the interaction under non–phase-separating conditions were followed as a function of the concentration of N (concentration of P was held constant—see Materials and Methods). The ratio of N and P in droplets was estimated according to

\[
r_D = \frac{N_D}{P_D} = \frac{(N_{TOT} - N_{OBS})}{(P_{TOT} - P_{OBS})} \quad (1)
\]

\(N_D\) and \(P_D\) refer to the apparent concentration of droplet-associated protein, \(N_{TOT}\) and \(P_{TOT}\) refer to the total concentrations of P and N,
and $N_{OBS}$ and $P_{OBS}$ refer to the apparent concentrations in the liquid phase that are observed by NMR. This analysis reveals a clear dependence of $r_D$ on the total concentration of N, varying by nearly a factor of 3 for total concentrations of N that vary between 7.5 and 75 µM (Fig. 5 and table S1). This dependence indicates that a simple two-state exchange mechanism cannot explain the experimental observation and suggests that at least two steps are necessary to describe the process of droplet formation.

**RNA colocalizes to MeV droplets and triggers nucleocapsid formation in guttula**

The functional role of MeV LLPS was examined by adding short, fluorescently labeled RNA decamers to samples containing droplets. Within seconds of adding the RNA, the fluorescence intensity of the droplets increased, starting from the perimeter (Fig. 6A and movie S2). This demonstrates that RNA localizes preferentially to the membraneless organelles where it presumably interacts with the N:P complex. After incubation for 1 hour at 37°C, the samples were examined using negative-stain electron microscopy, revealing the presence of assembled nucleocapsid-like structures (Fig. 6B).

To determine the rate of nucleocapsid assembly, we used real-time NMR. This approach has recently been used to observe the kinetics of assembly of nucleocapsid-like particles in the dilute phase (13). Nonfluorescently labeled RNA hexamers comprising six adenines were added to the droplet suspension comprising $^{15}$N-labeled N, resulting in the assembly of nucleocapsid-like particles. Assembly can be followed by detecting the appearance and increasing intensity of NMR signals from the P$_{1-50}$ peptide that is released upon assembly of the nucleocapsids (Fig. 6, C and D). Comparison of the rate of increase of the NMR signals corresponding to P$_{1-50}$ in the condensed and dilute phase (in the absence of P$_{304-507}$) unambiguously shows that nucleocapsid formation is accelerated in droplets at the same global concentration of N$_{1-525}$.

**DISCUSSION**

Nonsegmented negative-sense RNA viruses are known to form inclusion bodies comprising proteins involved in the replication machinery. The recent observation of colocalization of N and P of both rabies (31) and measles (32) into liquid-like membraneless puncta following infection suggests that this phenomenon may play a role in viral replication or transcription and that the phenomenon may be widespread throughout the Mononegavirales order and possibly related viruses. The formation of such membraneless organelles has been proposed to enhance reaction rates by concentrating interaction partners in a confined volume, for storage purposes or, in the case of viral organelles, to provide protection from the host immune system. Investigating the role of this poorly understood phenomenon is essential to understanding the viral cycle. Describing the physical mechanisms that underlie their formation and stability...
represents a significant challenge, particularly if one considers their potential as targets of viral inhibition. To provide molecular insight into these aspects, we aimed to recreate the phenomenon of LLPS involving MeV N and P proteins in vitro.

We first demonstrated LLPS of N and P under conditions where neither protein undergoes phase separation alone. Expression of independent domains and combinations of domains then allowed us to identify the regions of N and P that are necessary for droplet formation in vitro. This revealed that apart from the first 50 amino acids of P that chaperone N, the rest of the 300–amino acid disordered P TAIL is dispensable for LLPS, while the tetramerization domain of P, the hitherto uncharacterized disordered domain P LOOP, and the three-helix bundle XD are all essential. N cannot be purified in an isolated form, owing to the propensity of the protein to bind to cellular RNA and form nucleocapsid-like structures. We therefore purified a chaperoned form of N bound to the N-terminal peptide of P, the rest of the 300–amino acid disordered P TAIL is dispensable for LLPS, while the tetramerization domain of P, the hitherto uncharacterized disordered domain P LOOP, and the three-helix bundle XD are all essential. N cannot be purified in an isolated form, owing to the propensity of the protein to bind to cellular RNA and form nucleocapsid-like structures. We therefore purified a chaperoned form of N bound to the N-terminal peptide of P, which maintains N in a monomeric, unassembled form (N 0 P). LLPS only occurs when N 0 P comprising the full-length N protein, including both N CORE and the 125–amino acid disordered N TAIL, is present. Full-length N and P were also coexpressed, and the integral N 0 P complex was shown to undergo LLPS. Overall, these results verify that this minimal in vitro system reproduces the characteristic colocalization of N and P in membraneless organelles that has been observed in infected cells.

We also identified the interaction between the helical motif of N TAIL and XD to be essential for phase separation. These results align closely with data measured in vivo on cells infected with rabies (31) and more recently MeV (32). The N TAIL::P XD interaction has previously been associated with transport of the polymerase complex to the nucleocapsid before replication and transcription (10, 12, 38, 39). Here, we demonstrate that at least one role of this interaction involves the free-energy stabilization of LLPS. Mutation of a single residue (S491L) that is known to mediate the interaction between N TAIL and P XD, and to strongly affect replication in vivo (11), results in abrogation of LLPS.

Additional weak interactions between P LOOP and N are also shown to modulate the fluidity of the droplets, suggesting that polyvalency of N:P interactions plays a role in droplet formation. The importance of both disordered and folded, or partially folded domains in the polyvalent complex that stabilizes the MeV droplets invokes their possible roles as "stickers" and "spacers" (40), which assure the non-covalent binding and physical separation of interacting components, respectively. Nevertheless, the role of P LOOP appears to be more
complex than this as the impact of this interaction on the nature of MeV droplets suggests that it is capable of fulfilling both roles.

Using NMR spectroscopy, we are able to investigate the thermodynamic equilibrium between dilute and condensed phases in the presence of MeV LLPS by comparing the rate of increase in signal intensity from N TAIL and the decrease in intensity of signal from P LOOP and XD, as a function of the concentration of N. Assuming that all signal derives from the dilute phase (as evidenced by rotational and translational diffusion measurements) and that signal drop results uniquely from slow exchange with an invisible form of each protein in droplets, our observations suggest that the stoichiometry of N and P within droplets depends on the ratio of the two proteins in the mixture. As the experimental data cannot be explained using a simple one-step reaction, it is interesting to speculate on the nature of the second interaction. One possible explanation involves N interacting with P to form droplets, with additional recruitment of N to existing N:P droplets. Such a model would be in agreement with the critical presence of the tetramerization domain and the polyvalency of the four branched dynamic XD interaction sites, which may form a dynamic scaffold in MeV LLPS to which additional N could be recruited. Note that both P and N remain dynamic in the context of the droplets, as evidenced by the rotational diffusion measured by fluorescence anisotropy. The proposed role of P as a dynamic scaffold is supported by the observation of differential translational mobility of P within droplets with respect to N, as reported by FRAP. More experimental measurements on this system are required to better characterize these molecular processes.

Replication and transcription of negative-strand RNA viruses are thought to occur within inclusion bodies comprising N, P, and L. The colocalization of these proteins within liquid droplets may act to increase local concentration of the components of the viral replication machinery, thereby increasing replication efficiency. We have therefore investigated whether the liquid droplets formed upon mixing of N and P in vitro are capable of supporting nucleocapsid assembly, which is an essential part of the viral replication process. We previously demonstrated that assembly can be observed in the dilute phase, by adding RNA to monomeric N:P and using NMR spectroscopy and fluorescence anisotropy to monitor the rate of assembly (13). By adding RNA to a suspension of N:P organelles, we show that RNA molecules preferentially localize to the droplets and, once within the droplets, trigger assembly of nucleocapsid-like particles. Notably, the rate of assembly is significantly increased compared to non–phase-separating conditions, providing evidence that LLPS has the ability to enhance viral function and offering a first glimpse of these viral factories in action. This also indicates that the condensed nature of the droplets does not restrict the ability to assemble these very large superstructures and demonstrates that these minimalistic N:P droplets are sufficient to promote one of the essential processes of viral replication. In this context, we note that nucleocapsid structures were observed to be ejected from liquid droplets formed in cells infected with rabies virus (31).

Phosphorylation of P and N have both been reported to affect MeV function (41, 42) and, more recently, to influence the nature of liquid droplet formation in vivo (32). Although both N and P were purified from E. coli in their unmodified forms, the technology...
described here will allow us to investigate the impact of phosphorylation in future studies. In particular, the role of the 300–amino acid disordered PTAIL domain may become clearer as this comprises numerous phosphorylation sites but is not essential for LLPS. This experimental system will also allow us to investigate the role of host proteins that are thought to enter inclusion bodies and the viral proteins C and L.

In conclusion, we have demonstrated that purified N and P MeV proteins form liquid-like membraneless organelles upon mixing in vitro. We identify weak interactions involving intrinsically disordered domains of N and P that are implicated in this process and identify one interaction between the C-terminal domains as essential to phase separation. P protein appears to play a role as a multivalent scaffold stabilizing the droplets. The droplets exhibit both physical and functional features characteristic of paramyxovirus inclusion bodies: RNA colocalizes to the droplets and thereby triggers assembly of N protomers into nucleocapsid-like particles that encapsidate the RNA. The ability to reproduce mechanistic behavior of MeV factories in vitro will allow us to further examine the role of additional host and viral partners at the molecular level. The observation that similar puncta or inclusion bodies have been found for numerous negative-strand RNA viruses, including filoviruses and pneumoviruses, suggests that the observations made here may be of general interest in understanding the molecular basis of mononegaviral infection.

MATERIALS AND METHODS

Protein expression, purification, and mutagenesis

All P and N constructs were cloned into pET41c(+) between the NdeI and XhoI cleavage sites, where the XhoI site was ligated with a cleaved SalI site of the insert, yielding a construct with a C-terminal 8His-tag (5). All constructs were expressed in *E. coli* Rosetta TM(IDE3)/pRARE (Novagen) overnight at 20°C after induction at an optical density of 0.6 with 1 mM isopropyl-β-D-thiogalactopyranoside. N525 was cotransformed with P50 and purified in complex. Cells were lysed by sonication and subjected to standard Ni purification in 20 mM tris (pH 8.0) and 150 mM NaCl or 500 mM NaCl for XD containing constructs. The protein was eluted with 20 mM tris (pH 8.1), 150/500 mM NaCl, and 500 mM imidazole and then concentrated and subjected to size exclusion chromatography (SEC, Superdex 75/200) in 50 mM Na-phosphate (pH 6.0), 150/500 mM NaCl, and 2 mM dithiothreitol (DTT) buffer (NMR buffer). The P50N525, P50N405, P300N525, and P300N405 were produced as described previously (13, 14). Mutation of S491L in N and of 409VLK411→AAA in P were inserted by site-directed mutagenesis, and P50N525 (S491L), N460–525 (S491L), P304–507 (409VLK411→AAA), and P377–507 (409VLK411→AAA) were produced and purified in the same way as wild-type proteins.

Expression of unlabeled protein was performed in LB medium. Proteins labeled for NMR (15N and 13C) were expressed under the same conditions in M9 minimal medium. Cysteine mutations for fluorescence labeling were performed by site-directed mutagenesis (43). Briefly, the protein was incubated overnight with 10 mM DTT, then dialyzed against 50 mM Na-phosphate buffer (pH 7.0) and 150/500 mM NaCl in two steps, and mixed with 5× excess of maleimide dye (fluorescein or Alexa Fluor 594) solubilized in dimethyl sulfoxide. After 2 hours of incubation at room temperature, the labeling reaction was stopped by adding 5 mM DTT, and proteins were separated from the excess dye by SEC (Superdex 75/200) in NMR buffer. SDS-polyacrylamide gel electrophoresis (PAGE) gels are shown in fig. S1.

RNA

All RNAs were purchased from Integrated DNA Technologies (Leuven, Belgium), and all were terminated with OH at both 3′ and 5′ or with fluorescein amide at the 3′ end.

Droplet assembly and turbidity assay

P and N constructs were first mixed and then diluted with buffer; thus, the final NaCl concentration was 150 mM (unless different NaCl concentration is stated). Samples were incubated at room temperature for 5 min, and absorbance at 395 nm was read on a NanoDrop 2000c spectrophotometer (Thermo Fisher Scientific, Waltham, MA). An absorbance value higher than 0.1 was interpreted as a presence of phase separation in the system.

Microscopy imaging

Samples were imaged using either DIC or epifluorescence on an Olympus IX81 inverted microscope. Fluorescence signal was acquired using a 485(20)-nm (fluorescein) or a 560(25)-nm (Alexa Fluor 594) excitation filter combined with a 525(30)-nm or a 607(36)-nm emission filter, respectively. Excitation light (Metal-Halide X-Cite 120) and emission wavelengths are split using a 4x4M-B quadriplan dichroic mirror (Semrock). Oil immersion objectives 60× [Olympus PlanAPON60, 1.42 numerical aperture (NA)] or 100× (Olympus UPLFLN, 1.3 NA) were used for imaging, and a Hamamatsu Orca Flash4.0-V2 sCMOS (scientific Complementary Metal-Oxide Semiconductor) 2048 × 2048 camera was used for detection. The acquisition and data analysis were performed with Volocity (Quorum Technologies) software.

Fluorescence recovery after photobleaching

FRAP was measured using an Olympus IX81 spinning-disk confocal microscope equipped with a Yokogawa CSU1X spinning disk head and using diode-pumped solid-state lasers 488 nm (50 mW Coherent Sapphire) and 561 nm (100 mW Cobolt JIVE4). Emission is filtered through a 520(28)-nm filter for fluorescein. Acquisition is performed with an iXon Ultra EMCCD (Electron Multiplying Charge-Coupled Device) (Andor) 512 × 512 pixel camera. FRAP was restricted to a region of interest, defined graphically, with a galvanometric FRAP/PA device (Andor) using the same lasers used for imaging with AOTF (Acousto-Optic Tunable Filter) 15% and with a dwell time of 50 ms for 10 repeats before bleaching and with a 2-s interval for 40 repeats after. The laser power and the dwell time during bleaching were optimized to bleach 50% of the initial fluorescence intensity. Images were analyzed using FIJI and python plugin ImageFRAP (https://imagej.net/Analyze_FRAP_movies_with_a_Jython_script); the recovery was fitted with a single exponent.

Fluorescence anisotropy

Fluorescence anisotropy decays were measured using a Mini-Tau fluorescence lifetime spectrometer (Edinburgh Instruments, UK) equipped with a 450-nm picosecond pulsed diode laser and a TC2E electronics module for time-correlated single photon counting. Photons were detected using a high-speed photon multiplier tube (Hamamatsu H10720-01). The sample was excited with vertically polarized light, and parallel and perpendicular polarized fluorescence light was detected during 120 s sequentially. Histograms of photon
arrival times were generated for every measurement, and anisotropy
decays were calculated according to \( r(t) = \frac{I_{\text{par}}(t) - I_{\text{per}}(t)}{I_{\text{par}}(t) + 2I_{\text{per}}(t)} \), with \( I_{\text{par}}/I_{\text{per}} \) being the intensities of parallel/perpendicular polarized light per time unit and \( G \) being the correcting factor for
detection differences between the two detection pathways. The \( G \) factor was measured for each buffer condition using the free dye.

**Isothermal titration calorimetry**

Isothermal titration calorimetry was measured using iMicroCal
iTC200 at 25°C. The titration experiment was performed by adding
2 μL of aliquots of 500 μM P\text{XD} into the microcalorimeter cell filled
with 50 μM N\text{TAIL}. P\text{XD} was titrated through 20 injections with 180-s
intervals in between; the reaction mixture was continuously stirred
at 750 rpm. The titration curve was fitted to the experimental data
using the Origin version 7.0 software from MicroCal.

**NMR spectroscopy**

All experiments were acquired in NMR buffer at 25°C. The spectral
assignment of P\text{50N525} at pH 6.0 was verified on the basis of published
assignment at pH 7.0 (13) using \(^{13}\text{C}, \(^{15}\text{N}\)-labeled samples using a set
of BEST-TROSY triple resonance experiments.

The spectral assignments of \(^{13}\text{C}\) - and \(^{15}\text{N}\)-labeled P\text{377–457} and
P\text{457–507} constructs were obtained using sets of triple resonance ex-
periments correlating Ca, Cb, and CO resonances at a \(^1\text{H}\) frequency
of 600 MHz. NMRPipe (44) was used to process the spectra, and
automatic assignment was performed with the program MARS and
manually verified (45). Secondary chemical shifts were calculated
using the random coil values from refDB (46).

**Ensemble analysis of P\text{LOOP}**

The genetic algorithm ASTEROIDS selected representative ensembles
[fitting \(^1\text{H}, \(^{13}\text{C}, \(^{15}\text{N}\)-, and \(^{13}\text{C}(\text{Ca, Cb, and CO})\) backbone chemical shifts].
A 10,000-member ensemble of P\text{LOOP} was generated using the statis-
tical coil model Flexible Meccano (47), and 200-conformer ensembles
matching experimental backbone chemical shifts were selected
from this ensemble using the ASTEROIDS genetic algorithm as
previously described (48, 49).

**Calculation of apparent \(K_d\) values from NMR experiments
for the interaction between N and P\text{LOOP}/P\text{XD}**

\(^{15}\text{N}\) relaxation rates were obtained by sampling the decay of magnet-
ization (seven delays between 0 and 1.71 s). \(R_{\text{ip}}\) relaxation rates were
measured using seven delay times between 0.001 and 0.24 s. The
spin-lock field was 1500 Hz, and \(R_2\) was calculated from \(R_1\) and \(R_{\text{ip}}\),
considering the resonance offset. The residue-specific apparent \(K_d\)
values for P\text{377–457} and N were calculated from \(^{15}\text{N}\) relaxation as
described elsewhere (50). P\text{377–507} concentration remained constant
(100 μM), and N concentration varied from 0 to 140 μM. The rotatio-
tional correlation time of the complex was assumed to be equal to \(\tau_c\)
of P\text{50N525} alone and was estimated from its molecular weight.

\(^{15}\text{N}\) relaxation dispersion was measured at a P\text{457–507} concentra-
tion of 100 μM in the presence of 70 and 30 μM unlabeled N\text{TAIL} at
700 and 850 MHz and using 14 points at CPMG frequencies between
31 and 1000 Hz with a constant-time relaxation of 32 ms (51). Data
for nine residues and two fields were fitted simultaneously using
ChemEx (https://github.com/gbouvignies/chemex) and a two-state
exchange model. The population of P\text{457–507} bound to N\text{600–525} (\(p_b\)) and
the exchange rate (\(k_{ex}\)) were obtained from the fit, and the \(K_d\)
was calculated assuming a 1:1 binding stoichiometry. The error of
the calculated \(K_d\) value was estimated by error propagation from the
fitting error of \(p_b\) and assuming a 5% error in concentration deter-
mination of both proteins.

**NMR measurements of phase-separating samples**

\(^{15}\text{N}\)-labeled P\text{50N525} and P\text{304–507} were premixed at different ratios
and diluted in buffer so that the final NaCl concentration was 150 mM
and then loaded in a 3-mm NMR tube. \(^1\text{H}\), \(^{15}\text{N}\)-HSQCUs and \(R_{1p}\)
experiments were measured as described for non–phase-separating
samples. Pulsed field gradient NMR diffusion experiments were
measured using \(^{15}\text{N}\)-edited stimulated-echo diffusion experiments
using a 300-ms diffusion delay at 16 to 20 gradient strengths ranging
linearly from 0 to 0.55 T m\(^{-1}\) (52). \(^1\text{H}, \(^{15}\text{N}\)-HSQCUs used for the devel-
opment of the kinetic model were measured using both P\text{50N525} and
P\text{304–507} \(^{15}\text{N}\)-labeled samples at a constant P\text{304–507} concentration of 75 μM
and P\text{50N525} 5, 10, 20, 50, and 100%. The concentration of P\text{50N525}
and P\text{304–507} in the dilute and condensed phase was estimated on the basis
of intensities of 23 for P\text{50N525} and 9 for P\text{304–507} nonoverlapping and
noninteracting peaks, which were summed and normalized by the
intensities of the peaks in the spectra of the individual proteins. The
amount of each protein in droplets was then estimated by subtraction.
This allows us to estimate the concentration of droplet-associated N
and P in the sensitive NMR volume (but not in droplets as we do
not know the volume of droplets). We can then estimate the ratio of
N and P associated to droplets as a function of concentration of N.

**Nucleocapsid assembly by NMR**

Kinetics of P\text{50N525} (alone or in the presence of P\text{304–507} or P\text{377–507})
nucleocapsid assembly were followed using NMR spectroscopy using
the same procedure as previously described (13). Time resolution was
approximately 8 min. The assembly process was initiated by adding
six-nucleotide RNA (OH-A\text{6}-OH), and its time trace was followed by
measuring intensities of resonances from P\text{1–50} on SOFAST
HMQC spectra. Assembly rates were obtained by averaging intensities
of 18 peaks and fitting them with a biexponential function; mean
assembly rates were calculated as follows: \(k_1*α_1 + k_2*α_2\), where \(k_1\) and
\(k_2\) are rates, \(α_1\) and \(α_2\) are their amplitudes.

**Negative-stain electron microscopy**

The nucleocapsid-like particles used for negative-stain electron
microscopy were obtained by adding 50 μM RNA (HO-A\text{6}-OH) to
droplets formed by mixing 20 μM P\text{50N525} and 80 μM P\text{304–507} in
a final volume of 20 μL. Samples were incubated for 1 hour at 37°C,
then applied to the clean side of carbon on mica (carbon/mica inter-
face), and stained with 2% sodium silicotungstate (pH 7.0). Micro-
graphs were taken with a T12 FEI microscope at 120 kV and a
magnification of 30,000.

**SUPPLEMENTARY MATERIALS**

Supplementary material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/14/eaaz7095/DC1

View/request a protocol for this paper from Bio-protocol.

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