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REVIEW

Protein nanocondensates: the next frontier

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Abstract

Over the past decade, myriads of studies have highlighted the central role of protein condensation in subcellular compartmentalization and spatiotemporal organization of biological processes. Conceptually, protein condensation stands at the highest level in protein structure hierarchy, accounting for the assembly of bodies ranging from thousands to billions of molecules and for densities ranging from dense liquids to solid materials. In size, protein condensates range from nanocondensates of hundreds of nanometers (mesoscopic clusters) to phase-separated micron-sized condensates. In this review, we focus on protein nanocondensation, a process that can occur in subsaturated solutions and can nucleate dense liquid phases, crystals, amorphous aggregates, and fibers. We discuss the nanocondensation of proteins in the light of general physical principles and examine the biophysical properties of several outstanding examples of nanocondensation. We conclude that protein nanocondensation cannot be fully explained by the conceptual framework of micron-scale biomolecular condensation. The evolution of nanocondensates through changes in density and order is currently under intense investigation, and this should lead to the development of a general theoretical framework, capable of encompassing the full range of sizes and densities found in protein condensates.

Keywords Phase separation · Intrinsically disordered proteins · Protein condensates · Biomolecular condensates · Protein conformation · Protein folding · Membraneless organelles · Mesoscopic clusters, Nanocondensates, Protein coacervates · Protein colloids

Abbreviations

ALS	Amyotrophic lateral sclerosis
BR	Bodies bacterial ribonucleoprotein bodies
DLS	Dynamic light scattering
FTD	Frontotemporal dementia
IR	Insulin receptor
IDD	Intrinsically disordered domain
IGF	Insulin-like growth factor
MRG	Mitochondrial RNA granule
N-protein	Nucleocapsid protein
Pol II	RNA polymerase II
polyQ	Polyglutamine track
PRM	Proline-rich motif
RBP	RNA binding protein
SVs	Synaptic vesicles
SLS	Static light scattering
SG	Secretory granule
SH3	SRC homology 3
α-Syn	α-Synuclein
TEM	Transmission electron microscopy.

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Introduction

Protein conformation and condensation

The conformation of proteins results from a complex interplay of physicochemical forces acting on protein and solvent atoms to shape the conformational space. The conformation, in turn, endows proteins with the capacity to interact, with themselves and/or other macromolecules, and form assemblies. Protein assemblies come in multiple forms and sizes: stoichiometric complexes, open-end and closed oligomers, and a variety of molecular condensates. Indeed, the view of proteins as freely diffusible monomeric molecules is either

an experimental approximation or an ideal construction. In vivo, one always has to consider collections of physically associated and interacting protein molecules. Ultimately, both protein conformation and biological function can only be approached by considering proteins as social entities (Barrera-Vilarmau et al. 2022).

Protein condensates are dense phases composed of a large number of ordered and disordered protein domains interacting homo- and heterotypically. These dense phases exhibit a range of material properties, viscous liquids, percolated gels, amorphous solids, ordered solids, and crystals, with sizes from dozens of nanometers to some microns (Wunderlich 1999; Haas and Drenth 1999; Van Der Lee et al. 2014; Soranno 2020; Goetz and Mahamid 2020; Sawaya et al. 2021). Several recent and excellent reviews on the multiple aspects of protein condensation are available, for instance, Uversky (2017), Banani et al. (2017), Holehouse and Pappu (2018), Choi et al. (2020), Alberti and Hyman (2021), Lyon et al. (2021) Abyzov et al. (2022), Mohanty et al. (2022), and Vazquez et al. (2022).

In the last decades, a flurry of experiments highlighted the central role of biomolecular condensates in membraneless subcellular compartmentalization (Banani et al. 2017). In most cases, the main driving force for the formation of biomolecular condensates is the increased intermolecular affinity caused by the multiplicity of interaction motifs and/or conformational disorder (Li et al. 2012a; Hyman et al. 2014; Choi et al. 2020). The realization that intrinsically disordered domains (IDDs) favor phase separation is of paramount importance, because it establishes a link between conformational changes at molecular level and a mesoscopic property of the system. Conceptually, this link also leads to a new dimension in the protein folding theory, in which protein condensation can be thought of as the collective folding of large assemblies of protein molecules. In this expanded view, intramolecular folding is a single-chain disorder-to-order transition, and intermolecular folding is a multichain disorder-to-order transition (Miskei et al. 2020; Nassar et al. 2021; Vazquez et al. 2022; Barrera-Vilarmau et al. 2022).

Biological relevance of biomolecular condensation

In the cell, phase separation refers to a physical process that produces a macromolecular condensed phase immersed in a liquid dilute phase. This self-organizing and entropically unfavorable process results in the compartmentalization and concentration of biomolecules without the need for membrane confinement or elaborate transport processes. In turn, compartmentalization allows the spatiotemporal organization and regulation of myriads of simultaneous biochemical reactions and macromolecular interactions (Hyman et al. 2014; Shin and Brangwynne 2017; Holehouse and Pappu 2018; Mathieu et al. 2020; Lyon et al. 2021).

Cellular condensed phases are highly dynamic and far from equilibrium. Although all kinds of biomolecules can participate, proteins and nucleic acids are the major players in biomolecular condensation (Li et al. 2012a). Based on sequence similarity, it has been speculated that a large fraction of the proteome participates in biomolecular condensation (Vernon and Forman-Kay 2019; Hardenberg et al. 2020). A number of reviews have discussed phase separation as a new mesoscale organizational principle in cell biology, akin to compartmentalization by membranes (Holehouse 2018; Turoverov et al. 2019; Lyon et al. 2021; Feng et al. 2021).

Prominent examples of large, non-solid biomolecular condensates are the nuclear pore complexes, centrosomes, nucleoli, P granules, stress granules, germ granules, Cajal bodies, Balbiani bodies, nuclear A bodies, paraspeckles, and bacterial ribonucleoprotein bodies (BR-bodies) (Frey and Görlich 2007; Boisvert et al. 2007; Brangwynne et al. 2009; Updike et al. 2011; Machyna et al. 2013; Molliex et al. 2015; Patel et al. 2015; Protter and Parker 2016; Schmidt and Görlich 2016; Banani et al. 2017; Wang et al. 2018; Martin and Mittag 2018; Woodruff et al. 2018; Fox et al. 2018; Correll et al. 2019; Tiwary and Zheng 2019; Latonen 2019; Sawyer et al. 2019; Yoshizawa et al. 2020; Roden and Gladfelter 2021; Roden and Gladfelter 2021; Azaldegui et al. 2021).

Biomolecular condensates play an essential role in many fundamental cellular processes. The free diffusivity allows rapid changes in the composition of biomolecular condensates in response to signaling events, and their interactions are sensitive to environmental changes such as concentration, pH, and ionic strength. In addition, post-translational modifications of proteins—including phosphorylation, methylation, ubiquitination, and sumoylation—modulate the biophysical and biochemical properties of the condensates. This broad sensitivity to multiple factors endows condensates with the ability to integrate a variety of signals, thereby orchestrating signal transduction, transcriptional regulation, genome organization, immune response, cell adhesion, and protein trafficking, among other essential functions. (Giannattasio et al. 1975; Michael et al. 1987; Dodson and Steiner 1998; Maji et al. 2009; Banjade and Rosen 2014; Pattanayak et al. 2020; Banjade and Rosen 2014; Kienzle and von Blume 2014; Parry et al. 2015; Zihni et al. 2016; Su et al. 2016; Chong and Forman-Kay 2016; Nusse and Clevers 2017; Milovanovic et al. 2018; Sabari et al. 2018; Du and Chen 2018; Gibson et al. 2019; Snead and Gladfelter 2019; Schaefer and Peifer 2019; Schaefer and Peifer 2019; Zhao and Zhang 2020; Beutel et al. 2019; Chen et al. 2020; Schwayer et al. 2019; Rouaud et al. 2020; Zhao and Zhang 2020; Ditlev 2021; Botterbusch and Baumgart 2021; Lin et al. 2022; Parchure et al. 2022; Wu et al. 2022a).

Phase separation is often the first step in protein aggregation, and condensate aging frequently leads to liquid-to-solid

conversion and fibrillation. Fibrillar, gel-like and amorphous solid biomolecular condensates have been linked to an impressive number of diseases: amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), Alzheimer, Huntington, Parkinson, Creutzfeldt-Jakob, Kuru, familial insomnia, transthyretin familial amyloid polyneuropathy, type II diabetes, multiple myeloma, cystic fibrosis, neurohypophyseal diabetes insipidus, nephrogenic diabetes insipidus, spinocerebellar ataxia, Fabry, spinal bulbar muscular atrophy, sickle cell anemia, retinitis pigmentosa, Niemann-Pick, Gaucher, myofibrillar myopathies, and others (Lagier-Tourenne et al. 2010; Molliex et al. 2015; Patel et al. 2015; Wilkaniec et al. 2016; Kundra et al. 2017; Boeynaems et al. 2018; Klaips et al. 2018; Yadav et al. 2019; Babinchak et al. 2019; Ray et al. 2020; Mathieu et al. 2020; Aarum et al. 2020; McAlary et al. 2020).

Somatic mutations in oncogenes and tumor suppressors are a main driving force of cancer development. Aberrant biomolecular condensates and aggregates driven by gene mutation and fusion play a central role in this disease the second leading cause of death worldwide (Petronilho et al. 2021; Davis et al. 2022; Taniue and Akimitsu 2022).

Viruses resort to protein condensation for replication and progeny assembly. Viral condensates have been named viral inclusions, virosomes, viral factories, viroplasms, mininuclei, aggresomes, etc. (Wu et al. 2022b). Many of these condensates are connected to organelles: mitochondria (flock house virus); lysosome (Semliki Forest and rubella virus), peroxisome (tomato bushy stunt virus); Golgi complex (Kunjin virus); and ER (hepatitis C virus, dengue, severe acute respiratory syndrome coronavirus-2) (Etibor et al. 2021; Iserman et al. 2020).

Also, extracellular condensates are ubiquitous. These include collagen, blood clot forming fibrin, insect elastic matrix protein resilin, hinge ligament of bivalve mollusks, abductin, spider and insect silks, matrix proteins of squid suckers, attachment fibers and adhesives of mussels, and bacterial biofilms (Muiznieks et al. 2018; Urosev et al. 2020; Seviour et al. 2020; Yanagisawa and Davis 2010). Spider silk materials are semi-crystalline condensates of ordered domains containing spidroin (Walker et al. 2015; Malay et al. 2020). The paradigmatic example of crystalline condensates is the insulin-Zn^{2+} hexamer deposited in the secretory granule of β cells (Dodson and Steiner 1998; Kaissaratos et al. 2021).

The emerging concept of protein nanocondensation

Most of the biomolecular condensates described above were discovered and initially studied as bodies larger than the resolution limit of light microscopy. This is particularly true for condensates formed in liquid-liquid phase separation processes, such as membraneless organelles, which attract so much attention in cellular biology. Accordingly, much of the current conceptual framework of phase condensation has evolved from the analysis of large liquid-like condensates.

The large and easy-to-observe late products of phase separation are preceded by smaller condensates with sizes of tens to a few hundred of nanometers. The biological relevance of these nanocondensates—also referred to as mesoscopic clusters, nanoscale clusters, or nanoparticles—had long been suspected, but only recently, it was convincingly demonstrated (Georgalis et al. 1999; Gliko et al. 2005; Maes et al. 2015; Safari et al. 2015; Pan et al. 2010; Keber et al. 2021; Alberti and Hyman 2021; Pauly et al. 2023).

Nanocondensates are deemed important precursors in nucleation processes, such as crystallization, irreversible aggregation, and fibrillation; in turn, these nucleation processes characterize many physiological and pathological conditions (Vekilov 2004; Pan et al. 2010; (Sosa et al. 2016; Schubert et al. 2017; Chan and Lubchenko 2019; Toledo et al. 2019; Mudogo et al. 2020; Hondele et al. 2020; Choi et al. 2020; Xu et al. 2021; Yang et al. 2021; Petronilho et al. 2021).

The physicochemistry of nanocondensation in liquid solutions is much less understood than that of bulk liquid-liquid phase condensation. This review addresses past and present work on protein nanocondensation and attempts to foresee the evolution of this fascinating field of research.

Principles of protein condensation

Protein condensation is a phase separation phenomenon that occurs when the balance of interactions among biomolecules and between biomolecules and solvent leads to the demixing of the solution into two or more phases with different densities. Temperature, protein concentration, and chemical composition are the most important variables driving phase separation: enthalpy, entropy, and heat capacity are functions of temperature, concentration provides the spatial proximity of protein chains that stabilizes condensed phases, and chemical composition modulates intra- and intermolecular affinity. Upon cooling, demixing from saturated solutions may originate liquid condensates. Liquid condensates may transform into gels, ordered or amorphous solids, and crystals. In vivo, the different phases are far from equilibrium, and their separation is regulated by means of changes in the concentration and mutual affinity of their constituents. Concentration can be changed via protein synthesis, degradation, or transport, whereas affinity can be changed by post-translational modifications or changes in ionic strength, pH, or cofactors (Patel et al. 2015; Fox et al. 2018; Mukherjee et al. 2020; Hondele et al. 2020).

Although some features of the protein sequence and, especially, conformational disorder favor phase separation, any protein and any conformational state would condense, provided that the appropriate conditions for it are found (Lerman et al. 1966; Siezen et al. 1985; Thomson

et al. 1987; Broide et al. 1991; Berland et al. 1992; Martin and Mittag 2018; Hardenberg et al. 2020; Miskei et al. 2020; Horvath et al. 2020; Poudyal et al. 2022). The number of components further influences the density and material properties of the condensates. Simple temperature-concentration phase diagrams based on polymer chemistry principles can be used to describe the phase behavior of binary systems (Thomson et al. 1987; Broide et al. 1991; Berland et al. 1992; Muschol and Rosenberger 1997; Haas and Drenth 1999; Grouazel et al. 2006; Cardinaux et al. 2007; Dumetz et al. 2008; McManus et al. 2016; Shin and Brangwynne 2017; Adamcik and Mezzenga 2018; Holehouse and Pappu 2018; Peran et al. 2019; Yadav et al. 2019; Alberti et al. 2019; Soranno 2020; Dignon et al. 2020). However, more elaborate treatment are needed for multicomponent systems in the cell (Riback et al. 2020). In cellular environments, "scaffold" proteins with high propensity to phase-separate drive the condensation of "client" proteins (Banani et al. 2016). In turn, client proteins may pervade the multicomponent condensate or associate to its surface (Kelley et al. 2021).

Figure 1 shows a generic temperature–concentration phase diagram for proteins. Two regions are separated by the solubility (saturation) curve: the undersaturated and the supersaturated liquid regions. Liquid condensates, gels, amorphous aggregates, fibers, and crystals may emerge upon nucleation in the metastable, supersaturated region (Arakawa and Timasheff 1985; So et al. 2016; Vecchi et al. 2020; Noji et al. 2021). There is a strong connection between protein concentration and phase separation. Indeed, condensation can be seen as a way of increasing protein concentration above the solubility limits. In gels, amorphous aggregates, and fibers, the volume fraction of protein can be considered independent of temperature. Therefore, in Fig. 1, vertical lines mark the separation of these states. The binodal curve is the upper boundary of the region of metastable equilibrium between the single-phase and the liquid condensate. The spinodal curve is defined as the boundary between metastability and instability, and below it, due to the absence of a nucleation energy barrier, phase separation is instantaneous. The temperature ordinate tangent to the binodal defines the critical temperature (T_c), at which the protein concentration of the two separated phases is the same; i.e., it is the highest temperature that allows phase separation. Below T_c, each temperature ordinate intersects the coexistence curve at two concentration values, defining the concentrations of the dilute (light) and dense phase, respectively. The displayed phase diagram is that for a protein with upper critical-solution temperature, which is the most frequently observed. However, phase separations with lower critical-solution temperature, whose coexistence curve is a mirror image of that displayed, have been also characterized (Martin and Mittag 2018).

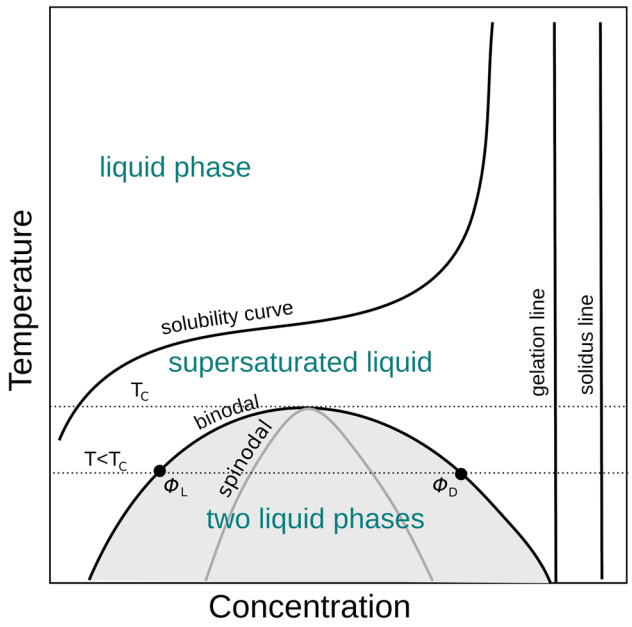

Fig. 1 Schematic protein-water phase diagram in the temperature–concentration plane. Below the solubility curve, supersaturated solutions are metastable and undergo transitions to the condensed liquid phase or to solid-like phases, such as gel states, crystalline states, amorphous solids, and fibers. The liquid-liquid binodal (coexistence curve) indicates the metastable boundary that maps the transition to the two-phase regime, where a liquid condensed phase coexists with a liquid dilute phase. The critical temperature (T_C) is the temperature at which the concentration differences between the two liquid phases vanishes and a homogeneous solution exists. At each temperature below T_C, the binodal defines pairs of protein concentrations, Φ_L and Φ_D, that characterize the protein volume fractions of the light and dense phases, respectively. The spinodal curve maps the boundary of the metastable regime. Below it, phase separation is abrupt and not limited by a nucleation energy barrier. The concentrations of the light and dense phases remain constant for a given temperature and are independent of the total protein concentration of the system, whereas the relative volumes of each phase change with the total concentration of the system. Thus, the left arm of the binodal maps the volume predominance of the light phase, whereas the volume of the dense phase predominates at the right arm. This explains why at low total concentrations, the system exhibits droplets of highly concentrated protein dispersed in a dilute phase, whereas at high total protein concentration, droplets of dilute protein are dispersed in a dense phase. The gelation line represents the protein concentration of gel phases. The solidus line defines the protein concentration of crystals, amorphous aggregates, and fibers

At each temperature, the concentration (density) of the two separated phases is thermodynamically constrained to the values on the coexistence curve. Concomitantly, mass conservation constrains the relative volume of the two separated phases in the system: on the right arm of the coexistence curve, a light phase is dispersed in the predominant, dense phase, whereas the opposite occurs on the left arm. The concentrations of the components are advantageously treated as volume fractions (Φ, the fraction of the total volume occupied by each component). By definition, $\Phi = C$

$\times V_s$, where C and V_s are the concentration and specific volume of the components, respectively. Unitless Φ values allow an easier appreciation of the intermolecular distances. The ideal, densest packing of spheres corresponds to $\Phi = 0.74$, value at which each sphere is at a contact distance with twelve nearest spheres. However, for entropic reasons, random hard-sphere packing of fluids corresponds to Φ values of approximately 0.5 (Manoharan 2015). In phase-separated liquid condensates, typical Φ values are approximately 0.3. In most protein crystals, Φ ranges 0.27–0.65 (Matthews 1968). However, in some extreme cases, crystal unit cells contain about 15% solvent and $\Phi = 0.85$. At $\Phi = 0.3$, the distance between protein molecules approaches the molecular diameter. As expected, the number of intermolecular contacts increases with Φ values and with the complimentary shape of the molecules (Lawrence and Colman 1993).

Flory–Huggins lattice models capture the enthalpic-entropic contributions to liquid condensation (Huggins 1941; Flory 1942). In the simplest form, the free energy in these models can be defined as

$$\Delta G = K_B T \left[\left(\Phi / V_e \right) \ln(\Phi) + (1 - \Phi) + \chi \Phi \, (1 - \Phi) \right],$$

where χ is a temperature-dependent parameter accounting for the strength of intermolecular interactions; V_e is a temperature-dependent, effective molecular volume corresponding to the phase separating component and relative to the solvent; Φ is the volume fraction of the macromolecule; and K_B is the Boltzmann constant. In this equation, the two left and the right terms between brackets represent the entropic and enthalpic components of ΔG, respectively (Curtis et al. 2001; Dumetz et al. 2008: Spruijt et al. 2010; Wolf et al. 2014; Brady et al. 2017; Wei et al. 2017; Martin and Mittag 2018; Ruff et al. 2018; Li et al. 2018; Shapiro et al. 2021). Since the entropy of mixing strongly disfavors phase separation, enthalpic terms tilt the balance toward the association (demixing) of separating molecules.

Clusters of a small number of molecules may form spontaneously by thermal fluctuations in saturated solutions. The growth in number and size of the clusters eventually leads to bulk phase separation. In classical nucleation theory, the free energy of cluster formation depends on the surface energy and on the difference in the chemical potential between the dense and dilute phases, which is negative in supersaturated solutions and positive in subsaturated solutions. These two terms scale with the square and the cube, respectively, of the radius of the cluster. A critical size exists, the nucleation barrier, at which the free energy is at a maximum. Below this barrier size, clusters tend to shrink, and above it, they tend to grow (Oxtoby 1992; Vekilov 2016). In subsaturated solutions, nucleation theory predicts that the probability of forming clusters larger than 3–5 molecules is essentially zero (Kar et al. 2022). To explain the existence of clusters in subsaturated solutions, non-classical, two-step nucleation theories were proposed, according to which a mesoscopic liquid condensate is formed first. Then, in a second step, the molecules within this liquid-dense nanocondensate may rearrange to form nuclei with different degrees of order. The mesoscopic cluster in the first step is considered a metastable intermediate, as its free energy is lower than in the initial homogeneous solution, but higher than in the following more ordered phase (Kashchiev et al. 2005; Vekilov 2010; Vekilov 2016; Zhang 2017; Wang et al. 2022).

Different types of nanocondensation

Nanocondensates in crystal nucleation

The link between nanocondensation and crystallization was first noticed in 1999 (Georgalis et al. 1999). One of the most studied proteins in this regard is lysozyme (Pan et al. 2010; Sleutel and Van Driessche 2014; Maes et al. 2015; Safari et al. 2017; Li et al. 2011; Li et al. 2012b; Vorontsova et al. 2015a; Nikfarjam et al. 2019). Other proteins closely investigated because of their tendency to form nanocondensates were hemoglobin (Galkin et al. 2007; Knee and Mukerji 2009; Safari et al. 2015), lumazine synthase, (Gliko et al. 2007), glucose isomerase, (Sleutel and Van Driessche 2014; Maes et al. 2015; Van Driessche et al. 2022), insulin (Kaissaratos et al. 2021), and ferritin (Houben et al. 2020). As a result of these studies, mesoscopic clusters of lysozyme, glucose isomerase, ferritin, and insulin became important models for protein crystal nucleation (Vekilov 2010; Sleutel and Van Driessche 2014; Zhang 2017; Houben et al. 2020; Kaissaratos et al. 2021; Van Driessche et al. 2022). Importantly, it has recently been shown for insulin that crystal nucleation by prior nanocondensation is much faster than direct nucleation from the solution.

Nanocondensates as precursors of fibers and solid-like aggregates

In sickle cell disease, a single point mutation in hemoglobin causes the formation of long pathological fibers, in a temperature-dependent manner. Differential interference contrast, UV resonance Raman spectroscopy, and dynamic light scattering (DLS) measurements evidenced that, in concentrated solutions, metastable clusters of mutated hemoglobin nucleate the formation of polymers (Pan et al. 2007; Galkin et al. 2007; Knee and Mukerji 2009).

Huntington's disease, a disorder that affects neurons, is characterized by the deposit of insoluble aggregates of pathological variants of huntingtin. These pathological variants have a polyglutamine track (polyQ) in the N-terminus

that is much longer than normal, causing a toxic gain of function (Walker 2007). A 78-residue, N-terminal segment of huntingtin including a 40-residue polyQ repeat was found to populate nanocondensates. A connection between the nanocondensates and the formation of toxic aggregates was established by showing that the destabilization of the nanocondensates leads to a reduction in aggregation (Posey et al. 2018).

The tumor suppressor p53 is a transcription factor whose inactivation by mutations is associated with almost all cancers (Levine 2019). The phase behavior of p53 and one of its mutants was thoroughly examined (Yang et al. 2021; Safari et al. 2019). In cellular studies, the combined staining with an antibody specific for misfolded or aggregated p53 and with the amyloid probe thioflavin T suggested that the p53 variant forms aggregates with narrow size distribution within the cytoplasm of breast cancer cells, whereas wild-type p53 does not. The p53 aggregates were visualized as puncta, with diameters compatible with nanocondensates. In dilute solutions, p53 also populated nanocondensates, which could be thoroughly characterized using biophysical techniques (Pedrote et al. 2020; Petronilho et al. 2021).

Freely diffusible nanocondensates

Since a large fraction of cellular proteins dwells on the edge of solubility (Vecchi et al. 2020; Poudyal et al. 2022), freely diffusible nanocondensates may be ubiquitous. In this regard, differential pressure filtration, size exclusion, and dilution experiments suggest that the cytoplasm may be organized into nanoassemblies (Li et al. 2012a; Keber et al. 2021; Alberti and Hyman 2021). The following examples of nanocondensates are not obviously restricted in their diffusion by cellular structures.

In a recent work, it was reported that subsaturated solutions of RNA binding proteins (RBPs) form heterogeneous nanoclusters below the concentrations at which typical bulk phase separation occurs (Kar et al. 2022). Also, it was reported that nanocondensates of MEG-3 deposit at the interface of *P* granules assembled by PGL-1 and PGL-3. Interestingly, surface adsorption of MEG-3 prevents the coarsening of the *P* granule without affecting PGL-3 exchange (Folkmann et al. 2021). In this regard, MEG-3 nanocondensates behave as Pickering agents, i.e., as nanoscale particles that adsorb to condensate interfaces and stabilize them. Importantly, these in vitro effects were related to the in vivo phase behavior of *P* granules during oocyte maturation and polarization. Thus, nanocondensates acting as Pickering agents may be another feature for biomolecular organization.

In living embryonic stem cells, mediator and RNA polymerase II (Pol II) form small transient clusters that associate with chromatin, have material properties of phase-separated condensates, and respond to transcriptional inhibitors. Mediator clusters have the size of typical nanocondensates. Pol II clusters co-condensates with mediator clusters, forming large (> 300 nm) and more stable clusters. This suggests that nanocondensates of mediator, transcription factors, and clustered enhancer elements interact with Pol II clusters in transcriptional condensates in vivo (Cho et al. 2018).

Nanocondensates may have important roles in metabolic control processes, such as proteostasis and regulated hormone secretion. Several secretory granule proteins have been associated with the formation of nanocondensates in granulogenesis and insulin secretion. Chromogranin B, ICA512 RESP18HD, insulin, and proinsulin nanocondensates were characterized in vivo and in vitro by different authors and hypothesized to be significant drivers in secretory granule biosynthesis (Bearrows et al. 2019; Toledo et al. 2019; Parchure et al. 2022; Rohli et al. 2022).

Membrane-associated nanocondensates

Membrane anchored condensates assemble at concentrations well below the saturation concentrations of liquid–liquid phase separation from homogeneous solutions. This feature considerably expands the range of biological functions of nanocondensation. Indeed, nanocondensation allows for precise localization of very dilute proteins, such as those involved in signaling, recognition, metabolic regulation, and genetic regulation. In addition, nanocondensates can exert forces on membranes and drive trafficking between different cellular compartments (Mitchison 2020; Ditlev 2021).

An excellent example of membrane anchored nanocondensates is that of the insulin receptor (IR), which forms dynamic nanocondensates at the plasma membrane, in the cytoplasm, and in the nucleus of human hepatocytes and adipocytes. The IR nanocondensates can be visualized as punctate bodies, and the material properties of these clusters of IR are influenced by physiological, pathological, and pharmacological stimuli. Importantly, the behavior of IR clusters may be associated with insulin resistance, which has implications in diabetic disorders (Dall'Agnese et al. 2022). Insulin and insulin-like growth factors (IGFs) trigger essential physiological mechanisms related to metabolism, differentiation, or growth. IR and IGF receptors act upon IGFs, mediating insulin/IGF signaling by recruiting a series of signaling factors into clusters with properties and characteristics expected of nanocondensates (Gao et al. 2022).

Synaptic vesicles (SVs) are membrane-bound bodies that contain neurotransmitters, and neurotransmission is a highly regulated process that depends on the precise spatiotemporal release of neurotransmitters. Such a process is made possible by the localization of tightly packed conglomerates of SVs anchored to the presynaptic plasma membrane. These micron-sized conglomerates contain hundreds of 40-nm

SVs. It has been proposed that the conglomerates of SVs are liquid condensates in which one of the components is a membrane-bound vesicle and the others are scaffolding proteins (Milovanovic and De Camilli 2017; Sansevrino et al. 2023). Remarkably, the SVs themselves are nanocondensates of neurotransmitters bound by a membrane.

Several other dynamic signaling condensates involved in the transmission of receptor activation information have been characterized to date (Wu 2013; Case et al. 2019). For example, the T-cell receptor activation causes the formation of nanocondensate compartments attached to the plasma membrane (Bunnell et al. 2006). Also, the nano-co-condensation of T-cell signaling components, phospho-LAT, Grb2, and Sos1, on the surface of artificial membranes is readily observed (Su et al. 2016; Ditlev 2021).

Within the mitochondrial matrix, newly synthesized RNA and RBPs form nanocondensates that appear as punctate subcompartments associated with membranes and are referred to as mitochondrial RNA granules (MRGs). The internal architecture of MRGs was investigated in vivo using fluorescence superresolution localization microscopy and found to consist of compacted RNA embedded within a protein cloud (Rey et al. 2020).

Biophysical properties of nanocondensates

Using DLS, static light scattering (SLS), and Brownian microscopy, several studies identified and characterized clusters of 30–100 nm in lysozyme solutions (Pan et al. 2010; Li et al. 2011, 2012b; Vorontsova et al. 2015a; Safari et al. 2015; Maes et al. 2015; Vorontsova et al. 2015b; Safari et al. 2017; Yamazaki et al. 2017; Byington et al. 2018; Nikfarjam et al. 2019). Important conclusions were drawn from these studies: (a) the size of the clusters was not very sensitive to changes in concentration and increased slowly with incubation time; (b) the changes in volume fractions with bulk concentration were unlike those seen in conventional amorphous aggregations, formation of amyloids, crystallization, or bulk liquid-liquid phase separations; and (c) lysozyme clusters were in apparent equilibrium with the solution (Li et al. 2012b; Safari et al. 2017). However, the notion that lysozyme mesoscopic clusters were in equilibrium with lysozyme monomers was disputed in a recent work (Nikfarjam et al. 2019), in which the authors concluded that nanocondensates of lysozyme consisted of irreversibly unfolded or damaged molecules that could be removed permanently by filtration through 20-nm pore filters. Regarding the material properties and conformational status, most studies concurred that nanocondensates of lysozyme were distinct from fibers and other amorphous aggregates formed by non-native or chemically damaged molecules. Quite the contrary, the nanocondensates of lysozyme were characterized as amorphous-solid or liquid-like collections of natively folded molecules (Vorontsova et al. 2015b; Maes et al. 2015; Safari et al. 2017).

The mechanism underpinning the early assembly and subsequent growth of lysozyme crystals has been thoroughly investigated. Since direct nucleation in solution is too slow to explain observed crystallization rates, two-step mechanisms were proposed, in which crystals are nucleated on heterogeneous surfaces or within previously formed nanocondensates (Vekilov 2016; Zhang 2017). By using time-resolved liquid-cell transmission electron microscopy to examine the nucleation of lysozyme crystals, it was possible to distinguish amorphous-solid nanocondensates that served as heterogeneous nucleation surfaces from presumably liquid-like nanocondensates that nucleate crystalline material inside (Yamazaki et al. 2017).

Another protein model for nanocondensation, sixty mers of lumazine synthase, was characterized by DLS, SLS, atomic force microscopy, and Monte Carlo simulations. Under conditions where no macroscopic liquid condensation exists, the lumazine particle was found to be an aggregate of about 350 nm (10^3 sixty mers) (Gliko et al. 2007). The authors concluded that lumazine nanocondensates are metastable not only with respect to the crystals but also with respect to the dilute solution. Moreover, the authors posited that mean cluster size is determined by the kinetics of growth and decay and not by thermodynamics.

Mesoscopic clusters of glucose isomerase observed by Brownian microscopy were of 300 nm diameter and remained stable in time. Furthermore, these clusters were in equilibrium with the solution and participated in crystal growth. However, cluster formation was significantly slower than cluster dissolution, and no cluster formation was detected below a critical protein concentration in the solution (Sleutel and Van Driessche 2014). Interestingly, these authors showed by laser confocal microscopy that 3D nucleation can be initiated by the fusion of the mesoscopic clusters of glucose isomerase with the macroscopic crystal surfaces. The same conclusion was drawn from experiments with crystals of lysozyme, proteinase K, insulin, xylanase, and triosephosphate isomerase. In a later study, aged solutions of glucose isomerase containing clusters of 500–1000 nm were analyzed by DLS, confocal depolarized DLS, and oblique illumination microscopy, and the results suggested that the mesoscopic clusters were liquid-like or amorphous solids and that they could locate crystal formation (Maes et al. 2015). However, a different conclusion was drawn from cryo-transmission electron microscopy experiments, in which metastable dense-liquid precursors of the crystalline state could not be identified, and the earliest precursors exhibited different degrees of crystallinity (Van Driessche et al. 2018) (Van Driessche et al. 2022). Furthermore, the effects of ions and crowding agents on the size and number

of glucose isomerase nanocondensates and of these in the crystallization process revealed multiple nucleation pathways (Wang et al. 2022).

Ferritin crystallization was followed in solution by time-dependent cryogenic scanning transmission microscopy. The images unveiled the initial formation of amorphous aggregates that undergo desolvation and lead to a structural evolution toward a final crystalline phase, which arises gradually via a continuous increase in order and density (Houben et al. 2020). The authors conclude that this nanocondensation mechanism is at odds with classical nucleation theory, which posits that full order and density emerge from the beginning in the primordial condensate and that the gradual evolution of order and density also provides insights beyond current models of non-classical crystallization.

Three different phases in equilibrated solutions of huntingtin were analyzed by electronic transmission microscopy (TEM) (Posey et al. 2018). According to the images, one of the two liquid phases comprised mainly monomers and oligomers, the other was enriched in nanocondensates of 25 nm in diameter and about 500 molecules in number, and the third phase was a solid fibrillar condensate. However, the only technique employed to measure the aggregates was TEM, and it would be desirable to confirm the above findings with complementary DLS and Brownian microscopy measurements.

Nanocondensates of the tumor suppressor p53 have been the subject of several biophysical studies. In dilute solutions, purified p53 formed nanocondensates that could be monitored by oblique illumination microscopy and DLS (Li et al. 2011) (Vorontsova et al. 2015b) (Vorontsova et al. 2016). At 15 °C, 220-nm filtered p53 solutions produced no microscopically traceable speckles, and particle sizes measured by DLS indicated radii corresponding to p53 tetramers and low-order oligomers. However, at 25 °C, nanocondensates of ~ 50 nm radius were detected, which grew in size with temperature reaching 145 nm at 37 °C. As well, particle number increased with temperature. The volume fraction of the aggregate increased with the initial p53 concentration, whereas the size of the individual particles remained constant. We estimate that nanocondensates of 50 nm radius would contain 103 loosely packed p53 tetramers. Compared to wild type, the disease-related p53 mutant R248Q exhibited an enhanced tendency to form nanocondensates: at 15 °C, R248Q p53 formed aggregates of 50 nm radius on average. The behavior of p53 mesoscopic aggregates contrasts with conventional liquid-liquid phase separation, in which, as the concentration in the whole system increases, the concentration of the bathing solution remains constant and the volume of the dense phase increases. A model based on the p53 results was proposed to explain the differences between nanocondensation and conventional normal liquid-liquid condensation. This model posits that (a) clusters form because of the accumulation of conformationally destabilized and misassembled oligomers, (b) clusters grow in size and number with temperature because of the partial unfolding of p53, and (c) mesoscopic clusters of p53 are transient formations in route to conventional liquid condensation, gelation, fibrillation, and amorphous precipitation. In this latter respect, p53 mutants form classical liquid droplets that turn more rapidly into solid-like aggregates compared with the wild-type protein (Pedrote et al. 2020; Petronilho et al. 2021).

The capacity of insulin to populate multimers, oligomers, and nanocondensates in vitro is well documented (Pekar and Frank 1972; Dodson and Steiner 1998; Nielsen et al. 2001; Attri et al. 2010; Jonassen et al. 2012; Landreh et al. 2012; Nilsson 2016; Xu et al. 2012; Kaissaratos et al. 2021; Karmakar et al. 2022; Silva-Jr et al. 2022). Contrastingly, the condensation of the insulin biological precursor, proinsulin, has been much less studied (Pekar and Frank 1972). Recently, using DLS, we observed that in the pH range of 7.3–5.4 and with or without micromolar concentrations of Zn^{2+}, proinsulin populate nanocondensates ranging in size from 15 to 300 nm (10^2–10^6 molecules). We also found that both proteins engage heterotypically in the formation of nanocondensates with other protein cargoes of the insulin secretory granule (Toledo et al. 2023). Based on our observations of in vitro nanocondensation, we hypothesized that proinsulin may condense in vivo and be a significant driver of secretory granule biosynthesis (Parchure et al. 2022; Bearrows et al. 2019; Rohli et al. 2022).

On the other hand, the nanocondensation of insulin in the SGs may nucleate insulin crystals. Growing evidence suggests that the formation of insulin crystals does not follow the classical nucleation theory but involves a two-step mechanism primed by nanocondensates. In the classical nucleation theory, crystals grow from sparse and ordered nuclei that are immersed in the solution and prefigure the morphology of the large crystals. In the two-step mechanism, nuclei grow out of dense and disordered nanocondensates, in which the high concentration helps to overcome the nucleation barrier making the process highly efficient (Sleutel and Van Driessche 2014; Zhang 2017; Kaissaratos et al. 2021).

The formation of nanocondensates from the interaction between the SRC homology 3 (SH3) and the proline-rich motif (PRM) domains—–two widespread modules that form tandem arrays in signaling proteins—was described by Li and coworkers (Li et al. 2012a). In this seminal work that first illuminated fundamental aspects of protein phase-separation and condensation, the authors showed cryo-electron microscopy images of droplets of about 200 nm in diameter formed by SH35 plus PRM5. A relation was found between the nanocondensation observed in vitro and in cells. The coexpression of fluorescently labeled repeat

SH3 and PRM domains in HeLa cells resulted in the formation of approximately 200–500 nm puncta that were not observed in cells expressing these domains separately or coexpressing shorter versions of the repeats, indicating that the formation of these puncta depends on the interaction between the two high-valency molecules. Both fluorescence signals in these bodies recovered within about 10 s after photobleaching, indicating that there was a rapid exchange of both components with the surrounding cytoplasm and suggesting that these nanocondensates have a dynamic liquid-like physical nature.

α-Synuclein (α-Syn) liquid condensation leads to irreversible amyloid fibril formation, a process that was related to Parkinson's disease. Using interferometric light scattering, DLS, and TEM, it was demonstrated that α-Syn forms nanocondensates, both above and below the critical concentration for phase separation (Ray et al. 2023). The formation of α-Syn nanocondensates containing tens to hundreds of molecules at physiologically relevant concentrations was very fast and preceded the formation of microscopically visible, conventional liquid condensates. However, below saturation concentration, α-Syn nanocondensates accounted for a very small volume fraction. The apparent mass and volume fraction of these nanoclusters increases with the protein concentration in the system, and the nanocondensates persisted above saturation concentrations, along with larger conventional droplets. Nanoscale α-Syn droplets could only be separated using ultracentrifugation and were of a liquid like nature (Ray et al. 2023).

Nucleocapsid protein (N-protein) packages the viral genome of SARS-CoV-2 into ribonucleoprotein particles. The 45-kDa N-protein is dimeric and possesses two folded domains with nucleic acid binding sites, flanked by intrinsically disordered domains. A thorough biophysical study of the condensation of the N-protein was recently carried out by Zhao and colleagues (Zhao et al. 2021). In this study, hydrodynamic, spectroscopic, and calorimetric methods were applied to assess size, composition, solution structure, and thermodynamic stability of the N-protein and its complexes with oligonucleotides of different lengths $(GT)_n$. At low concentrations, neutral pH, moderate salt concentration, and temperature, the N-protein was partially disordered and in a monomer-dimer equilibrium, without evidence of higher order condensation. The solution behavior of the N-protein changed dramatically upon binding to oligonucleotides, forming oligomers and higher order condensates. Nanocondensation was readily evident for complexes of 3 μM N-protein with $(GT)_6$ and $(GT)_{10}$. Under these conditions, DLS experiments showed the formation of nanocondensates of 200 nm and incipient microscopic phase separation. Notably, the free N-protein underwent a temperature-induced nanocondensation, populating 200-nm particles above 50 °C. In the case of the N-protein complexes, the 200-nm nanocondensates underwent a transition above 40 °C leading to micron-sized condensates.

The kinetics of nanocondensation in supersaturated solutions of the prion-like low-complexity domain of hnRNPA1 was characterized by time-resolved X-ray scattering. The kinetic experiments were combined with equilibrium studies to conclude that, at the mesoscopic scale (> 100 nm), these nanocondensates behaved as predicted by classical nucleation theory, whereas smaller condensates deviated strongly from that theory, leading to large effects on nucleation rates. Thus, the molecular details of the nanocondensates must be taken into account to accurately describe the kinetics of phase separation. These results question whether a single theoretical framework can be used to appraise the kinetics and equilibrium of condensates of any size, from oligomers to nanocondensates to microscopic condensates (Martin et al. 2021).

The size distribution, morphology, and abundance of nanocondensates of RNA binding proteins from the FUS-EWSR1-TAF15 family in solutions subsaturated for phase separation were investigated in an enlightening work by Kar and colleagues (Kar et al. 2022). They found by DLS analysis that these proteins formed heterogeneous distributions of clusters in subsaturated solutions: while the predominant species were small clusters, the distributions were heavily tailed toward larger nanocondensates in the 100–700 nm range. Furthermore, the size of the nanocondensates increased as the bulk concentration approached 2 μM, the saturation concentration. Importantly, the size of the nanocondensates decreased upon dilution and increased with concentration in the subsaturated regime. This later feature points out the reversibility of the nanocondensation and the liquid nature of the FUS nanocondensates. Using TEM and nanoparticle tracking analysis, the authors also showed that the nanocondensates of FUS had roughly spherical morphologies and accounted for 1% of the protein molecules. In addition, the authors found that nanocondensates grew into micron-scale bodies above the saturation concentration and that nanocondensation could be decoupled from macroscopic phase separation by additives such as 1,6-hexanediol or ATP, which are known to suppress phase separation and dissolve micron-scale condensates.

The examples given in the previous section clearly show that nanocondensation is difficult to fit into classical models of protein aggregation and phase separation. The main conflicting points are the following: nanocondensation can take place in undersaturated protein solutions, in unexpected regions of the conventional phase diagrams (Fig. 1); the size and volume fraction of the nanocondensates are not always correlated with bulk protein concentrations; and the physical properties of nanocondensates correspond to various types of macroscopic condensates, for example, dense liquids amorphous and ordered solids (Arakawa and

Timasheff 1985; Pan et al. 2010; Sleutel and Van Driessche 2014; Zhang 2017; Safari et al. 2017; Kaissaratos et al. 2021; Kar et al. 2022; Martin et al. 2021).

These conflicting points uncover that condensation in subsaturated solutions cannot be approached from classical macroscopic phase-separation models based on supersaturation and a single energy scale, such as that in the Flory theory. Instead, in the subsaturated regime of the phase diagrams, reversible nanocondensations might be an extension of polymerization models based in the isodesmic association of molecules. However, how this oligomerization leads to sizes of thousands of molecules and hundreds of nanometers challenges conventional thinking based on chemical equilibrium. Indeed, the classical nucleation theory posits that subsaturated solutions should be devoid of clusters with more than a few molecules (Kar et al. 2022).

The polymorphism of nanocondensates poses an additional challenge to the search for a general theory in the formation of separate phases and the condensation of the molecules. Nanocondensates are distinct from other common protein condensates such as crystals, amyloid fibrils, gels, and amorphous precipitates (Safari et al. 2019; Yang et al. 2021). Accordingly, specific mechanisms for reversible nanocondensation have been proposed (Safari et al. 2017; Chan and Lubchenko 2019).

Concluding remarks

Interest in protein condensation has grown dramatically in the last decade, and the appeal of this topic lies in its direct relationship to a fundamental organizational principle of biological function, membraneless compartmentalization, which allows the integration and spatiotemporal coordination of cellular processes. Conformation endows proteins with the ability to interact with themselves and with other macromolecules to form condensates, and both protein conformation and biological function can only be fully understood by considering proteins as social entities with varying degrees of order and density.

In this review, we addressed the emerging concept of nanocondensation, a stage of the protein condensation process that typically involves 10^4 to 10^5 molecules and can occur in the absence of a higher order condensation. The formation of nanocondensates is at the origin of phase separation and generates the different material states of proteins, including dense liquids and a variety of amorphous and ordered solids.

The study of nanocondensates is just beginning. In the past, the very idea of nanocondensation was scarcely accepted; nowadays, nanocondensation is a well-established fact. Perhaps the most remarkable feature of nanocondensation is its persistence under very dilute conditions, which is of the utmost relevance for cell physiology. Most proteins pervade the cell at submicromolar concentrations and will never reach the millimolar concentrations needed for the generation of microscopically observable phase separation. Thus, nanocondensation provides a way to achieve cellular compartmentalization in the scale of the hundreds of nanometers.

Nanocondensates can nucleate higher order condensations. The nucleation of the different material states of proteins is the subject of intense investigation, and thus, there is a wealth of experimental data that need to be interpreted in terms of general theories. From the analysis of the multiple examples in this review, it is clear that we are in the need of a general theoretical framework of protein condensation applicable to the whole scale of protein order, size, and density. At the lower end of the scale, protein assemblies of hundreds of molecules and sizes of a few nanometers can form stoichiometric complexes, open-end and closed oligomers, and random clusters. These small assemblies can be conveniently treated within the framework of chemical equilibrium and binding energies. At the upper end of the scale, protein assemblies of billions of molecules and sizes of microns can form bulk, phase-separated dense liquids, gels, aggregates, and crystalline materials. For dense liquids, general polymer theories, based on generic bulk intermolecular interaction parameters, provide a convenient framework for thermodynamic treatment. To fill the gap between the two extremes of the scale, we need a proper theory accounting for the assembly of 10^4 to 10^5 molecules. The new theory should explain the persistence of nanocondensates in subsaturated solutions and their further evolution into larger bodies with different degrees of order and densities.

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