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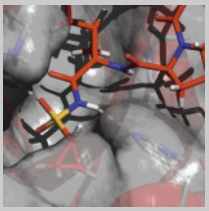

Medicinal Chemistry and Chemical Biology Highlights

Division of Medicinal Chemistry and Chemical Biology

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Phase Separation as an Emergent Concept in Genome Organization and Beyond

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Cells contain vast numbers of proteins, nucleic acids and metabolites that interact and react with each other during critical cellular processes, including gene regulation, cell signaling, or metabolism. On a sub-cellular level, processes are compartmentalized into different organelles, generally delimited by lipid membranes. On an even smaller scale, additional compartmentalization takes place in the form of membraneless compartments.[1] The most well-known subcompartment of this type is the nucleolus, where ribosome biogenesis takes place.[2] Cytoplasmic examples include stress granules or centrosomes that organize the microtubule network.[3] The size of those compartments ranges from 100 nanometers to a few micrometers and they all contain high concentrations of various proteins and nucleic acids that are assembled non-stoichiometrically. This allows the compartmentalization of biochemical reactions, such as signaling cascades or complex gene-regulatory processes, resulting in increased efficiency due to the high concentrations and proximity of all the involved factors.[4] To explain the underlying physico-chemical principles involved in the assembly of such compartments, phase separation processes have been proposed as a key driving mechanism.[1,5] As the formation of membraneless compartments plays a fundamental role in the organization and regulation of the cyto- and nucleoplasm of living cells, the study of phase separation processes represents a rapidly moving field in current biological research.

Basic Principles of Biomolecular Phase Separation

Initial observations showed that P-granules (RNA and protein-rich compartments in *C. elegans* embryos) and mammalian nucleoli exhibit liquid-like characteristics: They behave like oil-in-water droplets, fuse upon contact, wet the surrounding cellular structures, and rapidly condense and de-condense.[6] Importantly, fluorescence-based observation of protein diffusion revealed rapid molecular dynamics in all these subcompartments.[7] Liquid–liquid phase separation (LLPS) provides an intriguing theoretical framework to explain these observations. On a fundamental level, LLPS describes the demixing of multicomponent solutions when one or more solutes reach a critical saturation concentration (Csat) (Fig. 1a), reminiscent to the behavior of immiscible solvents such as oil and water. The driving forces of this process are favorable self-interactions between the molecules in the condensed phase, compared to less favorable interaction with the solvent or other solutes. If the energy difference between these interactions balances out the entropy cost of condensate formation, phase separation occurs.[8] In LLPS, the condensed phase remains highly dynamic and shows liquid-like behavior, as opposed to a gel-state, crystal or aggregate. Importantly, many proteins have been shown to undergo phase separation *in vitro*. This ability has generally been associated with the presence of intrinsically unstructured regions (IDRs) that can interact *via* transient non-specific interactions.[9] However, LLPS is not only mediated by non-specific contacts but also by specific binding domains within proteins, provided that the interactions are transient, dynamic, and multivalent.[1,9a] The combination of specific binding domains ('stickers') connected through unstructured 'linkers', often found in scaffold proteins *e.g.* in signal transduction,[9a] gives rise to the 'sticker-linker' paradigm of LLPS (Fig. 1b).

Phase Separation in Genome Organization

In the cell, the situation is naturally more complex and condensates contain many different macromolecules, including various proteins and nucleic acids. Recent work has identified several subcompartments, which resemble phase-separated droplets, in particular within the nucleus where the DNA is organized as chromatin, a nucleoprotein complex with nucleosomes as its basic unit (Fig. 1c). Arrayed like beads on a string, nucleosomes

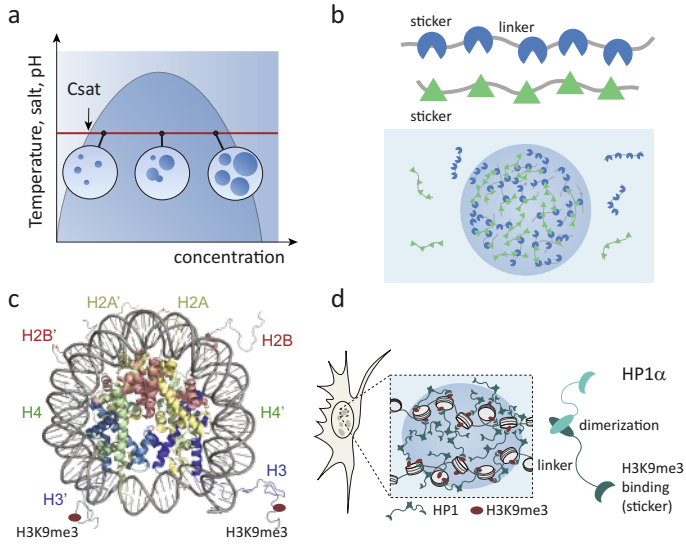

Fig. 1. a) Phase diagram for LLPS for a single-component solution. Component concentration changes along the red line; above Csat under the curve, molecules separate into two phases. b) Macromolecules containing sticker domains separated by linkers can phase separate through multivalent interactions. c) Structure of a nucleosome (PDB: 1KX5) with indicated histone modification H3K9me3. d) Model how dimeric HP1α can drive heterochromatin phase separation.

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form long fibers whose three-dimensional structure contributes to gene regulation: Depending on the activity of the underlying genome, the structure of chromatin is either open and accessible in transcriptionally active regions ('euchromatin'), or compact and transcriptionally inactive ('heterochromatin'). Within eu- and heterochromatin, the chromatin fiber exhibits preferential self-interaction, organizing DNA on a genomic scale.[10]

Recent *in vitro* experiments have revealed that nucleosomes and chromatin fibers can undergo phase separation to form dense 'chromatin droplets'.[11] Post-translational modifications (PTMs) of the histone proteins can modulate the degree of self-interaction within chromatin (Fig. 1c). For example, histone acetylation, involved in gene activation and thus found in euchromatin, disrupts key contacts between nucleosomes, resulting in the opening of chromatin structure[12] and the dissolution of phase-separated chromatin compartments *in vitro*.[11] Conversely, transcriptionally repressed and compact heterochromatin is associated with tri-methylation of H3 at lysine 9 (H3K9me3, Fig. 1c).[13] This modification serves as a binding site for heterochromatin protein 1 α (HP1α), a key factor in establishing a compact chromatin state.[14] HP1α is a constitutive dimer containing a H3K9me3 binding domain (playing the role of a 'sticker') and a charged, unstructured 'linker' connecting the functional domains.[15] Intriguingly, HP1α has been found to readily phase-separate *in vitro* and in cells.[16] Based on these findings, a model for heterochromatin formation was proposed, where H3K9me3-modified chromatin fibers partition into phase-separated HP1α droplets. The HP1α phase boundary delimits these repressive chromatin compartments and restricts biochemical access to the gene expression machinery,[16] generating a membraneless heterochromatin compartment responsible for gene repression (Fig. 1d). Individual HP1α proteins remain dynamic within these droplets, in particular in early fly embryos, but the condensates mature and adapt a more gel-like state in later development stages.[16b]

However, phase separation is not the only model which can explain the observed processes in cells: In an alternative model, HP1α associates with chromatin and cross-bridges chromatin strands through multivalent histone contacts, forming compact chromatin globules devoid of phase separation.[17] The dominant mechanism at play is thus still under investigation and might depend on cell type and development status.

Beyond the nucleolus and heterochromatin, phase separation may play a role in the formation of additional nuclear subcompartments: To activate transcription, transcription factors bind their target sites within enhancer regions in the genome, which are often separated from the genes under their control by thousands of base pairs. Moreover, groups of genes are often co-regulated by the same transcription factors.[18] Phase separation of proteins involved in transcription regulation brings distant regulatory elements together with promoters.[19] Such domains of active transcription within the genome may increase transcription efficiency and enable the co-regulation of gene groups. DNA repair processes represent another example relying on subcompartment formation. Upon DNA damage, the installation of post-translational modifications, such as polyADP-ribosylation, results in the formation of DNA repair foci which show phase-separated behavior.[20] Similarly, repair proteins can induce condensate formation, thereby concentrating repair factors for efficient DNA repair.[21]

While phase separation has been proposed to increase biochemical efficiency *via* concentration of factors and compartmentalization of reactions, it may also lead to insoluble aggregation and thus is implicated in diseases. A key example is the RNA binding protein FUS, which is involved in regulating many processes in gene expression, and the TAR DNA binding protein 43 (TDP43). Aggregates of these proteins, containing specific mutations, have been found in cell inclusions in amyotrophic lateral sclerosis (ALS) patients.[22] Similarly, repeat expansion mutations such as in Huntington's disease can result in the deposition of protein aggregates[23] resulting in neurotoxicity. Pharmaceutic interference with liquid-to-solid transitions of phase-separated protein compartments might thus present potential therapeutic options to treat such diseases, but successful intervention relies on the molecular understanding of the underlying processes.

New Tools to Investigate Phase Separation Processes

Gaining mechanistic insight into phase separation processes in cells, and discriminating LLPS from other possible mechanisms, is a difficult task. *In vitro* model systems allow to directly probe the propensity of macromolecules to undergo phase separation under highly controlled conditions. However, results from these studies cannot directly be extrapolated to the complex environment in the cells, where condensates may contain hundreds of different molecules. A more fundamental challenge to the concept of phase separation being a general mechanism for cell organization comes from the question of specificity: how can the cell simultaneously maintain a large number of different condensates with highly distinct compositions? Due to these difficulties, the generality of the phase separation concept as a key cellular organizing principle is a subject of ongoing debate.[5,24]

Thus, new tools are urgently required to identify, quantify and control LLPS processes, in particular in living cells. In recent years a plethora of novel approaches has been developed. As many proteins driving phase separation are natively unstructured, NMR methods provide unique insight into dynamic and low-affinity interactions and have thus been successfully employed to investigate proteins in condensates.[25] Similarly, single-molecule fluorescence methods enable the study of diffusional processes within phase-separated domains and, using energy transfer methods, enable a structural characterization of the involved IDR-containing proteins.[26] Fluorescent probes can also provide insight into the physico-chemical environment within subdomains in living cells. Fluorophores which are non-fluorescent but photoconvert to a fluorescent state when immersed in an environment of lower solvent polarity can serve as environmental probes[27] (Fig. 2a). When coupled to peptides which target phase-separated compartments, for example heterochromatin domains (Fig. 2b), the local environmental polarity of distinct compartments can be determined within cells and compared to similar protein phases *in vitro*[28] (Fig. 2c). Methods to directly control the phase separation of proteins in living cells enable the investigation of underlying processes. Chemical and optogenetic, or light-induced, means of modulating protein–protein interactions provide a rapid and graded control of protein phase separation within the complex cellular environment.[29] Finally, cryo-electron tomography of phase-separated domains in cells will help reveal any internal structure and organization on a molecular level.[30] Together, these methods will be instrumental in the coming years to dissect the mechanisms and functional consequences of phase separation in cell biology, and determine the generality of these processes across different cell types. Future quantitative insights into this intriguing biological field thus require a genuinely interdisciplinary approach combining diverse methods, including cell and molecular biology, biophysics and chemical biology.

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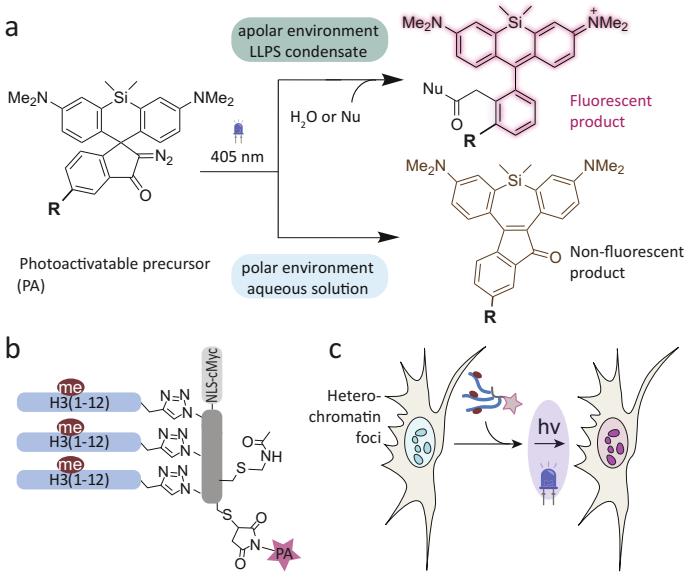

Fig. 2 a) Photochemistry of photoactivatable silicon rhodamine fluorophore, leading to different photoproducts depending on the polarity of the environment. b) Design of peptide reagents targeting heterochromatin compartments, carrying modified histone sequences tri-methylated at lysine 9, a photoactivatable sensor dye (PA), and a nuclear localization sequence (NLS-cMyc). Moreover, an Acm-protected cysteine handle can be used for further functionalization, *e.g.* with a cell-penetrating peptide. c) Heterochromatin staining using sensor peptides. The degree of fluorescence after photoactivation can be used to determine the polarity of the environment with the targeted subcellular domains.

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