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Solution NMR spectroscopy for investigation of liquid-liquid phase separation

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Abstract Liquid-liquid phase separation (LLPS) of biomolecules, a newly-found phase behavior of molecules in the liquid phase, has shown to its relationship to various biological function and misfolding diseases. Extensive studies have increasingly revealed a general mechanism of LLPS and characterized the liquid droplet; ho wever, intermolecular interactions of proteins and structural states of LLPS-inducing proteins inside of the droplet remain largely unknown. Solution NMR spectroscopy has emerged as a powerful approach as it provides invaluable information on protein intermolecular interactions and structures at the atomic and residue level. We herein comprehensively address useful techniques of solution NMR including the effect of paramagnetic relaxation enhancement for the study on the LLPS and droplet based on recent studies.

Keywords droplet, intrinsically disordered region, liquid-liquid phase separation, low-complexity proteins, paramagnetic effects, solution NMR spectroscopy

Introduction

One of the most emerging and novel recent topics in life sciences is liquid-liquid phase separation (LLPS) in which biomolecules including proteins and nucleic acids self-assemble to form liquid droplets in solution. LLPS has drawn increasingly attention as it is deeply responsible for a variety of essential biological events such as gene expression1, signal transduction,[2,3] stress response[4] and immune response.[5] Many of the cellular membrane-less organelles including nucleolus[6] and heterochromatin[7] in nuclei as well as balbiani body[8] and P granule[9] in cytosol are found to be formed by LLPS. Disease-related phase transition from the liquid droplet to insoluble amyloid fibrils has been also revealed.[10,11]

LLPS is a physical process and makes literally protein solutions separated. Two solution phases, a protein-less (diluted) phase and a protein-rich (dense) phase, form following LLPS (Figure 1). LLPS keeps fluid environments even in the dense phase of droplets, suggesting that the droplet is formed through weak and dynamic intermolecular interactions. Droplet formation is triggered by a lot of

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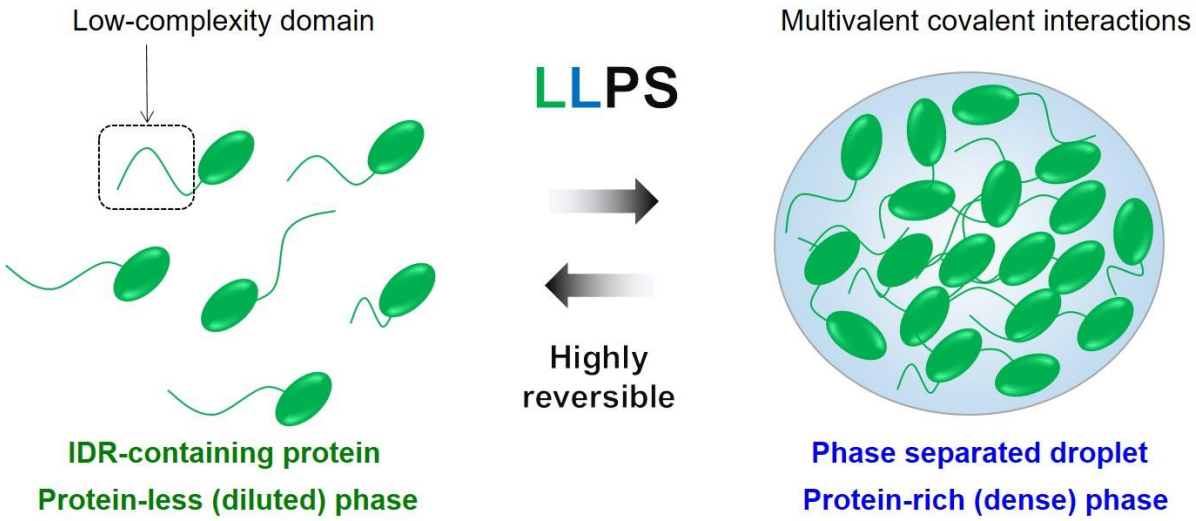

Figure 1. Liquid-liquid phase separation. Proteins including a low-complexity domain with an intrinsically disordered region (IDR) in the diluted solution phase (left) undergo highly reversible liquid-liquid phase separation (LLPS) to a protein dense phase through multivalent covalent interactions.

environmental factors and highly reversible. Of note, the proteins involved in the formation of the LLPS droplet often contain intrinsically disordered region (IDR) or the low- complexity (LC) domain.[12] Unlike other structural domains, The LC domain consists of highly-skewed amino acid composition, and is sometimes enriched in glutamine or proline residues. The functional importance of the LC domains has not been clear yet, however, recent studies have unveiled that LC domains are responsible for intermolecular association such as oligomerization to drive LLPS.[12,13] By taking advantages of LLPS, the proteins in droplets selectively recruit molecules such as DNA and RNA for intercellular function. Thus, structural states and intermolecular network in the liquid droplets are likely considered to play a key role for function.

Several physical properties to understand the fundamental principles and consequences of LLPS and droplet have been suggested based on few biophysical approaches. Microscopic tools have been frequently used to investigate the LLPS droplet. For example, fluorescence recovery after photobleaching (FRAP) assay (Figure 2) can observe a translational diffusion property of the molecule in the droplet.[2] However, detailed mechanisms of LLPS and physicochemical and structural natures of proteins in the LLPS droplet have been still poorly understood, mainly due to the scarcity of high-resolution structural information. In this regard, solution NMR spectroscopy is one of the most powerful tools to investigate molecules in the crude and crowded environment of the LLPS droplet at the atomic and residue level. In this mini-review, we exclusively highlight the several key applications of NMR to the study on proteins in the LLPS droplet and introduce promising NMR techniques.

Preparation of the condensed phase for the solution NMR study

At lower concentrations of salt, LC proteins often undergo the phase separation into μm-size droplets *in vitro*. In general, the droplet has extremely high concentrations of proteins compared to those in typical *in vitro* experiments: LC domains of FUS[14,15] and hnRNPA2[16] are concentrated to 120 mg/mL (~7 mM) and 440 mg/mL (~30 mM), respectively, upon the formation of the droplet. Despite the highly-concentrated states, the droplet still keeps liquid nature, which allows droplets to fuse each

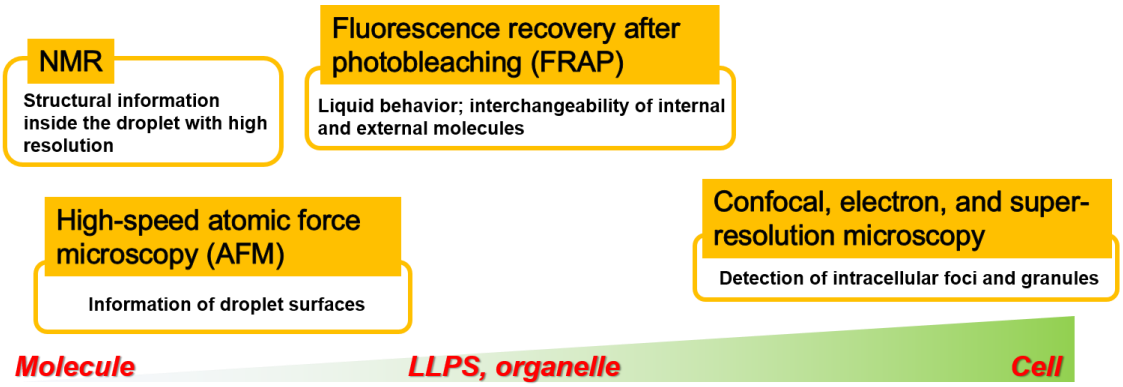

Figure 2. Biophysical technologies to study the LLPS and liquid droplets. Several approaches for the LLPS study from the molecule to the cell hierarchy are shown. NMR and high-speed AFM are powerful tools for detecting droplets at the atomic and molecule levels, respectively. Liquidity of droplets can be characterized with FRAP. Imaging methods based on various microscopy to visualize foci and membrane-less organelles are also displayed.

other. Thus, the phase-separated droplets can be collected into a single separated macroscopic phase by centrifugation, which are proper samples for NMR experiments.[14,15] In addition, by collecting the condensed phase, one can easily avoid observing of the resonances from the diluted phase (outside of the droplet). It is important to bear in mind that, although droplet formation concentrates proteins, even a small amount of proteins in the diluted phase prevent the selective observation of proteins inside the droplet as NMR resonances from proteins inside a droplet become broad and insensitive.

NMR observation of the proteins in the condensed phase

Inside of the LLPS droplet is a highly viscous environment. It has been reported that the diffusion of the FUS LC domain in the LLPS droplet is ~500 times slower than the diluted phase.[15] Although the restricted molecular motion in the condensed phase is obviously disadvantageous to the NMR observation, relatively sensitive NMR resonances of the protein in the LLPS droplet have been reported so far. Dynamic aspects of IDR of LC proteins that are devoid of the stable tertiary structure as well as highly-concentrated states in the droplet are advantageous to observing NMR signals. Decreases in the intensity of NMR peaks coming from viscous droplets can be compensated by increases in the intensity of NMR peaks due to local flexibility of IDR. Thus, several NMR studies have successfully reported that LC proteins keep disordered in the droplet.

NMR techniques to examine the protein structure and interaction in the condensed phase

Few approaches based on solution NMR have been used to investigate the structure and intermolecular interaction of proteins in the condensed phase. It has been reported that nuclear Overhauser effect (NOE) observation for FUS LC in the condensed phase. In order to selectively observe intermolecular NOEs,[15] (^{1}H)^{13}C HSQC-NOESY-^{1}H^{15}N HSQC experiments were performed using ^{12}C, ^{15}N-labeled FUS LC and ^{13}C, ^{14}N-labeled FUS LC (mixing with 1:1 ratio). Although solid evidence for intermolecular contacts in the condensed phase has been revealed, the limited resolution of the spectra, owing to the narrow dispersion of the resonances as well as low complexity sequence of proteins, hampered detailed residue-by-residue analyses. Exploitation of the segmental labeling should be effective for improved

resolution in the future studies.

Paramagnetic probes have shown to provide important structural information of LC proteins in the condensed phase. For the effective paramagnetic effects, a paramagnetic center, *e.g.*, nitroxide spin label, and a paramagnetic metal ion, *e.g.*, trivalent lanthanide ions, can be introduced to a protein of interest by using a chemical modification, *i.e.*, disulfide bond formation through a cysteine residue.[17] One of the advantages of the paramagnetic probe is in obtaining of long-range structural information among proteins. The paramagnetic center induces a variety of the paramagnetic effects including paramagnetic relaxation enhancement (PRE) and pseudocontact shift (PCS). PREs provide distance information within the range of < 25~30 Å from the paramagnetic center while PCSs give us distance and angular information within the range of < ~40 Å. The overview of the paramagnetic effects for the protein NMR study is comprehensively explained.[18]

Another advantage of the paramagnetic probe is that one can readily distinguish the intermolecular interaction from the intramolecular interaction (Figure 3). By mixing isotopically-labeled proteins, which are tagged with a paramagnetic center, with excess amounts of isotopically-unlabeled proteins without a tag, the paramagnetic effects only report intramolecular interactions among proteins including conformational states. Likewise, however, with a reverse way, the addition of excess amounts of isotopically-unlabeled proteins with a tag of a paramagnetic center to isotopically-labeled proteins without a tag exclusively provide information of intermolecular interactions including the oligomerization

It is noteworthy that PREs is markedly useful for the structural analysis of LC proteins.[15,16,19] Paramagnetic effects are detected more effectively with two-dimensional (2D) NMR spectra as 2D spectra inherently provide better resolution of NMR resonances of proteins than one-dimensional NMR spectra. Thus, even for LC proteins which show a narrow distribution of NMR resonances, the paramagnetic effect provides valuable residue-by-residue structural information as shown in the previous reports.[15,16,19] Of note, PRE is sensitive to study the minor population, which is in equilibrium, *i.e.*, an exchange process, with the major population.[20] Although this approach helps us characterize the minor conformational states in the LLPS droplet, one should keep in mind that the significant PREs are not always derived from the major conformational states. Introduction of other useful NMR techniques and their mutual combination will be highly beneficial for obtaining information on structural states and intermolecular interactions. In addition to the PRE approach, exploitation of the other paramagnetic effects including PCS and residual dipolar coupling (RDC) will further extends the possibility of the NMR methodology for the LLPS-related study

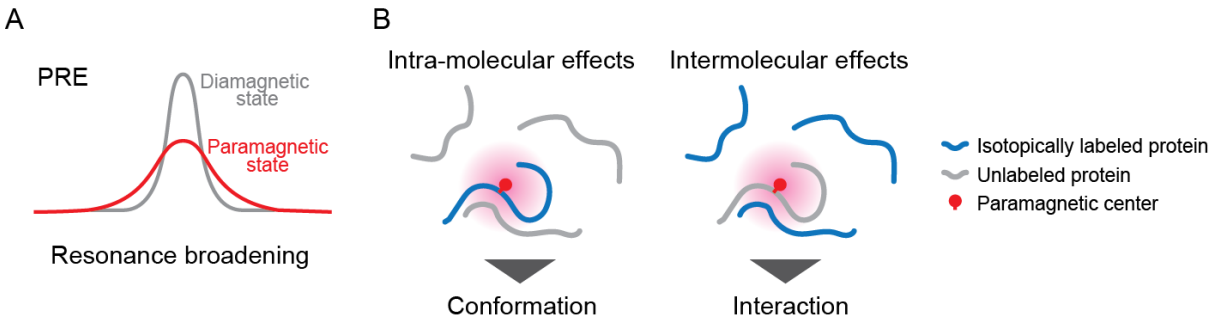

Figure 3. Paramagnetic relaxation enhancement (PRE) for the study on LLPS and droplet. (A) Schematic representation of PRE. PREs are generated by nitroxide spin labels or lanthanide ions and proportional to r^{-6} where r is a distance between the paramagnetic center and NMR-active nuclei. (B) Selective observation of intermolecular and intramolecular paramagnetic effects. A strategy to detect not only intra and intermolecular interactions but also protein conformational states in droplets is shown.

Conformational heterogeneity of the proteins in the condensed phase

It is still questionable which kinds of interactions mediate intermolecular contacts and contribute to the formation of the LLPS droplets although it is expected to be highly heterogeneous inside the droplet in terms of protein structures.[21] A lot of types of non-covalent interactions have been proposed, for instance, electrostatic, dipole–dipole, pi–pi, cation–pi, hydrophobic, and hydrogen bonding interactions,[13,15,21-23] and their multivalent interactions are a major play to construct the LLPS droplet. Heterogeneous features of the droplet are an obstacle to investigate intermolecular interactions in droplets at high resolution. Although NMR investigation might be suffered from the heterogeneity, NMR is still powerful to characterize molecules inside the droplet at the atomic resolution. We expect a new method based on solution NMR spectroscopy and a combination of other technologies such as high-speed atomic force microscopy (HS-AFM) as well as confocal, electron, and super-resolution microscopy (Figure 2) for the study on the LLPS droplet by overcoming issues including heterogeneity.

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