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Integrative structural biology of protein-RNA interactions in four dimensions

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Protein-RNA complexes underlie essential cellular processes and understanding their functional mechanisms requires structural analysis. Yet, their inherent flexibility, multi-valency, and dynamics make them challenging targets for structural biology. Integrative approaches, combining nuclear magnetic resonance (NMR), small-angle scattering, cryo-electron microscopy (cryo-EM), cryo-electron tomography (cryo-ET), crosslinking mass spectrometry, single-molecule techniques, and artificial intelligence (AI)-based predictions, have enabled the characterization of increasingly complex ribonucleoprotein (RNP) assemblies, *in vitro* and in their cellular contexts. These strategies have begun to capture molecular architecture and dynamic behaviors across time and space, paving the way for 4D structural biology. Here, we review recent developments in integrative modeling of protein-RNA complexes, highlighting advances in in-cell, 4D and condensate structural biology, and discuss how these approaches shape our understanding of RNP assembly, regulation, and function in physiologically relevant environments.

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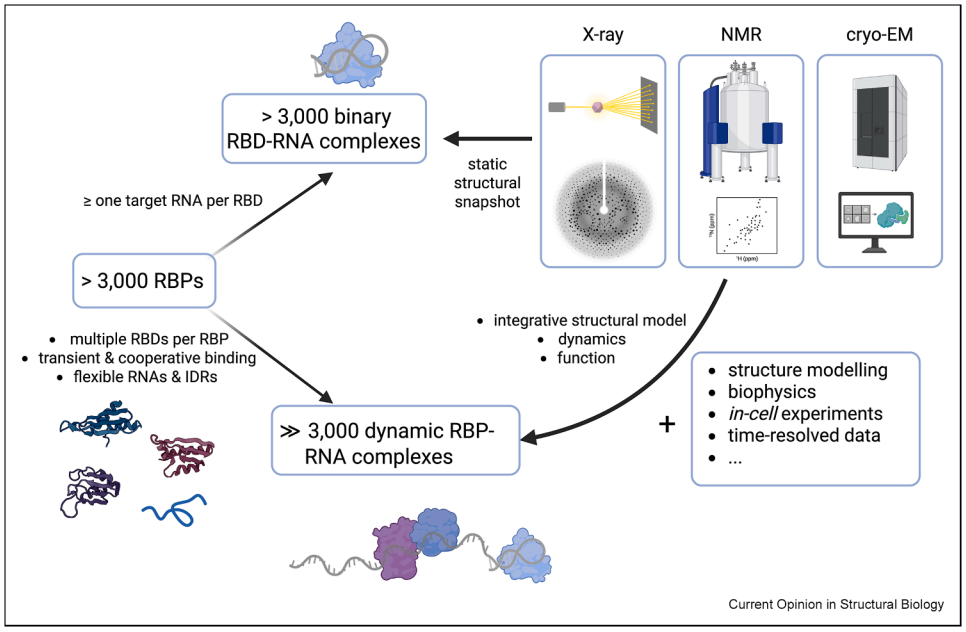
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RNA-binding proteins (RBPs) mediate RNA processing, folding, and stability, and are essential components of ribonucleoprotein (RNP) complexes that maintain cellular function. RNA interactome captures have greatly expanded the catalog of RBPs, revealing proteins to be RBPs although they do not feature a classical RNA-binding domain [1,2]. The lastest estimates identify more than 3000 RBPs within the human genome [3,4], some of which might engage in transient, non-sequence-specific RNA interactions. These studies also highlighted that intrinsically disordered regions (IDRs) constitute a large fraction of RBPs and frequently contribute to RNA recognition, RNP assembly, and condensate formation. Moreover, a single specific RBP is often involved in more than one RNP or function, increasing the possible number of interactions to be studied immensely. The sheer number of possible interactions to be studied poses a great challenge to structural biology, especially because the dynamic nature of RNPs, containing inherently flexible RNA molecules and protein IDRs, requires approaches that go beyond the classical single-technique methodology (Figure 1).

Integrative structural biology of protein-RNA complexes

Many protein-RNA interactions occur within very large macromolecular assemblies, such as the ribosome [5], the spliceosome [6] or the telomerase holoenzyme [7,8], whose structures have been extensively characterized by cryo-electron microscopy (cryo-EM) during the past decade. This single-method approach remains relevant for protein-RNA complexes as illustrated by recent structures of e.g. the ribosomal pre-initiation complexes, spliceosome recycling and other snapshots of telomerase [9–13]. However, these molecular machines are relatively rigid assemblies. For many other RNPs, transient interactions and cooperative binding between proteins containing multiple RNA-binding domains (RBDs) with intrinsically disordered extentions and inherently flexible RNA molecules give rise to dynamic and context-dependent assemblies [2,14]. Such systems are impossible to fully capture and understand using static structures obtained by a single method like X-ray crystallography or cryo-EM and require an integrative approach using complementary solution methods that can probe conformational dynamics. Here, we understand the term integrative structural biology in its classic sense as the integration of different kinds of restraints, derived from a variety of structural and biophysical methods, to obtain a structural model and/or information on the dynamics of the complex under investigation [15]. We do not consider the validation of a structural model by cellular mutational analysis to be integrative structural biology.

Figure 1

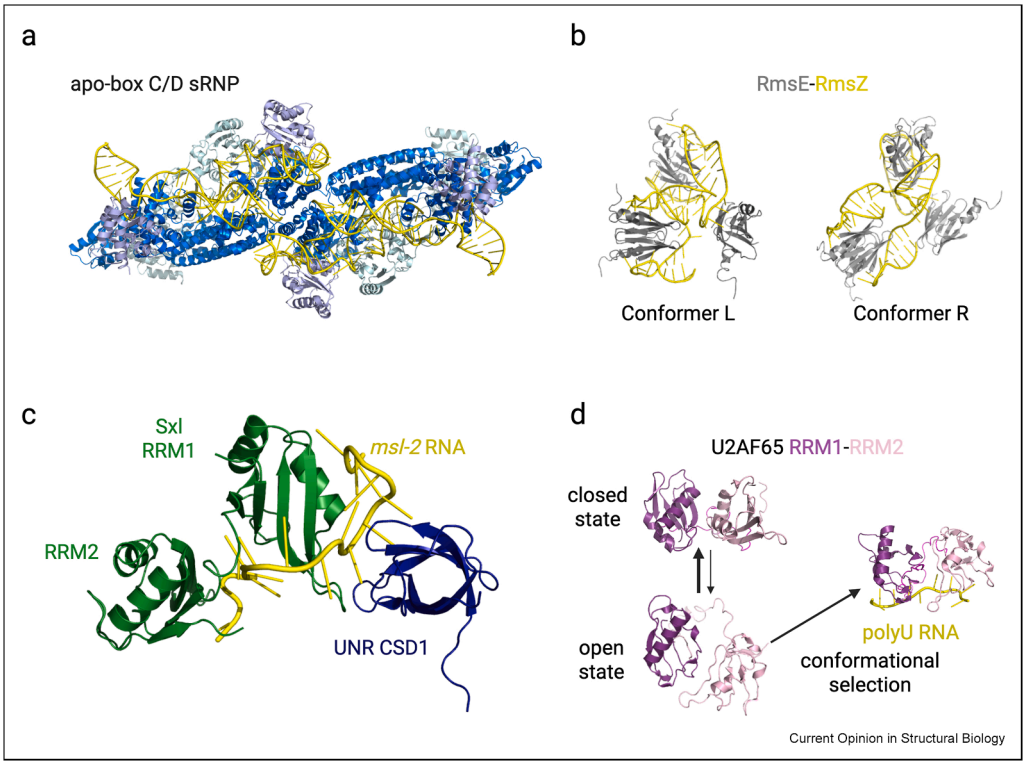


Integrative structural biology of protein-RNA complexes. Proteome-wide studies have identified more than 3000 RBPs. This results in >3000 binary RBD-RNA interactions if only one RNA target and one RBD per RBP is considered. However, many RBPs contain multiple RBDs and IDRs that mediate transient and cooperative interactions with several RNA targets, or bind cooperatively to RNA, giving rise to manifold more multi-component complexes comprising several RBDs, RBPs and/or RNA molecules. The classical structural biology methods (X-ray crystallography, NMR spectroscopy, and cryo-EM) can yield static structural snapshots of binary RBD-RNA complexes or larger rigid molecular machines. However, the combinatorial use of multiple, often flexible or flexibly connected RBDs and the dynamic nature of RNA give rise to a much larger number of dynamic and context-dependent RBP-RNA assemblies, which are not easily tractable with a single structural biology method alone. Integrative structural biology approaches that combine structural methods with complementary techniques such as biophysical measurements, in-cell experiments, and time-resolved analyses are therefore required to capture not only the structures, but also the dynamics and functions of these complex systems. Created in https://BioRender.com. RBPs, RNA-binding proteins; RBD, RNA-binding domains; IDRs, intrinsically disordered regions; NMR, nuclear magnetic resonance; cryo-EM, cryo-electron microscopy.

Nuclear magnetic resonance (NMR) spectroscopy has proven particularly valuable for studying protein-RNA interactions, as it readily provides at least residue-level information on structure and dynamics and yields structural ensembles that better reflect the conformational heterogeneity of flexible RNPs [16,17]. However, conventional NMR approaches are typically limited to relatively small complexes. Consequently, integrative structural biology strategies that combine multiple experimental techniques are often required to obtain comprehensive models of larger protein-RNA assemblies. These approaches integrate classical structural biology methods (X-ray crystallography, NMR spectroscopy, and cryo-EM) with complementary techniques and computational modeling to describe both the architecture and dynamics of large biomolecular complexes.

However, as of 01 June 2026, only 0.2 % of all entries in the Protein Data Bank are annotated as determined by integrating different methods. Of these, 26 are protein-nucleic acid complexes, and only two of them are protein-RNA complexes. This shortage is partly because hybrid models are very difficult to deposit to the PDB. This has improved to some extent by the recent launch of the Protein Data Bank - Integrative or Hybrid Modeling (PDB-IHM) archive and extensions to the PDBx/mmCIF standard [18]; consequently, most integrative models were not or only partially available from public repositories or not appropriately annotated as such. The major reason for the scarcity of integrative models, however, is the lack of a one-solution-for-all protocol: The best choice of methods needs to be adapted to each single case [19], which is a very difficult undertaking that requires access to and expertise in multiple high-end technologies. Therefore, only a few protein-RNA complexes have been characterized using such integrative strategies, including combinations of NMR with small-angle scattering [19,20], or electron paramagnetic resonance (EPR) [21]. Prime examples remain the 390 kDa archaeal box C/D ribonucleoprotein enzyme bound to RNA (Figure 2a, methly-trosy NMR and SAXS/SANS) [20], the non-coding RNA RsmZ protein sponge (Figure 2b, NMR and EPR) [21], the

Figure 2



Example structures of protein-RNA complexes obtained by classical integrative modeling. The prime examples of integrative structural biology remain **(a)** the structure of the 390 kDa archaeal box C/D ribonucleoprotein enzyme bound to substrate RNA, modeled by integrating crystal structures with NMR-derived paramagnetic relaxation enhancement (PRE) restraints and SAXS/SANS data (PDB 4BY9) [20] and **(b)** the non-coding RNA RmsZ acting as a protein sponge (PDBs 2MF0 and 2MF1) [21]. A larger assembly was modeled by integrating EPR data (no PDB entry is available). **(c)** The crystal structure of the cooperative complex of *D. melanogaster* Sxl RRM1,2 and Unr CSD1 with *msl*-2 RNA was confirmed in solution by SAXS/SANS and NMR techniques (chemical shift perturbations and residual dipolar couplings, PDB 4QQB) [22]. **(d)** U2AF65 discriminates strong and weak pY-tracts during splicing by conformational selection. This was revealed by integrating NMR (PREs), SAXS, and known crystal structures (PDBs 2YH0 and 2YH1) [23,25]. Created in https://BioRender.com. NMR, nuclear magnetic resonance; EPR, electron paramagnetic resonance.

Sxl-Unr-*msl-2* mRNA complex (Figure 2c) [22], the U2AF-RNA complex (Figure 2d, validated by SAXS, SANS, and NMR) [23–25], and the Brat-Pum-Nanos-*hb* mRNA complex, for which no PDB entry exists due to the dynamic nature of the assembly probed by methyl-trosy NMR, ^{15}N spin relaxation, small-angle neutron scattering and crosslinking mass spectrometry (XL-MS) [26]. More recently, a mechanistic model of cooperative RNA recognition by the antiviral RBP TRIM25 was derived from the integration of NMR, SAXS, and XL-MS data [27].

Specialized NMR methodologies further extend the size range accessible to solution studies probing structure and dynamics [28,29]; for instance, methyl-group and fluorine NMR have recently been used to probe interactions and dynamics in the 410 kDa eukaryotic RNA exosome complex [30].

A combination of cryo-EM, cryo-ET, XL-MS, and structural modeling advanced our understanding of mRNP nuclear export by the transcription-export (TREX) complex [31,32].

Earlier integrative strategies for protein-RNA complexes have been reviewed extensively elsewhere (e.g., Refs. [19,33,34]). Therefore, we next focus on recent studies that integrate emerging experimental and machine-learning approaches to model protein-RNA assemblies that remain difficult to characterize using structural biology techniques alone.

Artificial intelligence-based structure prediction for integrative modeling of protein-RNA complexes

Since the introduction of AlphaFold2 [35], AI-based protein structure prediction has transformed structural biology and increasingly supports integrative approaches for modeling macromolecular assemblies. The growing importance of integrative methods is reflected by the formation of research infrastructure consortia [36] and

the integration of integrative or hybrid models (IHM) into the Protein Data Bank through the PDB-IHM archive and extensions of the PDBx/mmCIF data standard [18].

More recently, AlphaFold3 [37] introduced the capability to predict RNA structures and protein-RNA complexes. However, reliable modeling of RNP assemblies remains challenging, largely due to the limited number of experimentally determined protein-RNA complex structures available for training. As discussed elsewhere [38,39], current predictions of RNA structures and protein-RNA interfaces are therefore less reliable than those for proteins alone, and predictions can be misleading rather than guiding research. Consequently, despite its considerable potential, AI-based structure prediction has not yet accelerated structural studies of protein-RNA complexes to the same extent as protein complex structure determination. Most RNP systems still require custom integrative strategies that combine computational predictions with complementary experimental data.

Integrative in-cell structural biology

Advances in cryo-ET have enabled the determination of biomolecular structures within their native cellular environment, allowing the integration of spatial and contextual information. A prime example, although not including RNA, is the combination of cryo-ET with cXL-MS data, AI-based structure prediction, and molecular dynamics simulations, which facilitated near-complete modeling of the nuclear pore complex (NPC) [40,41]. A recently developed integrative modeling workflow then modeled the NPC assembly pathway during mitotic exit on a spatiotemporal trajectory, using live-cell correlated cryo-ET, bulk fluorescence correlation spectroscopy-calibrated quantitative live imaging, and a structural model of the fully assembled nuclear pore complex [42].

The term 'integrative in-cell structural biology' in the context of protein-RNA complexes was coined by studies that combined *in situ* cryo-ET with whole-cell XL-MS to determine the architecture of the *Mycoplasma pneumoniae* expressome at subnanometer resolution [43,44]. The combination of cryo-ET and whole-cell proteomics was also used to obtain atomic models of mumps virus nucleocapsids bound to RNA inside infected cells, elucidating how cellular stress reactivates persistent infections [45]. In addition, cryo-ET proved capable of resolving *in situ* structures of human translating ribosomes, revealing how the translation-inhibiting drug homoharringtonine alters translation dynamics [46].

These approaches exemplify how *in situ* experimental and computational data can be integrated at the cellular scale to model biomolecular assemblies in their native environment.

4D structural biology – integrating temporal information

Complementary techniques, particularly single-molecule methods, are increasingly applied to probe the temporal dynamics of protein-RNA interactions, moving the field toward 4D structural biology. Integrating time-resolved data with structural snapshots allows the modeling of biomolecular processes on a temporal scale.

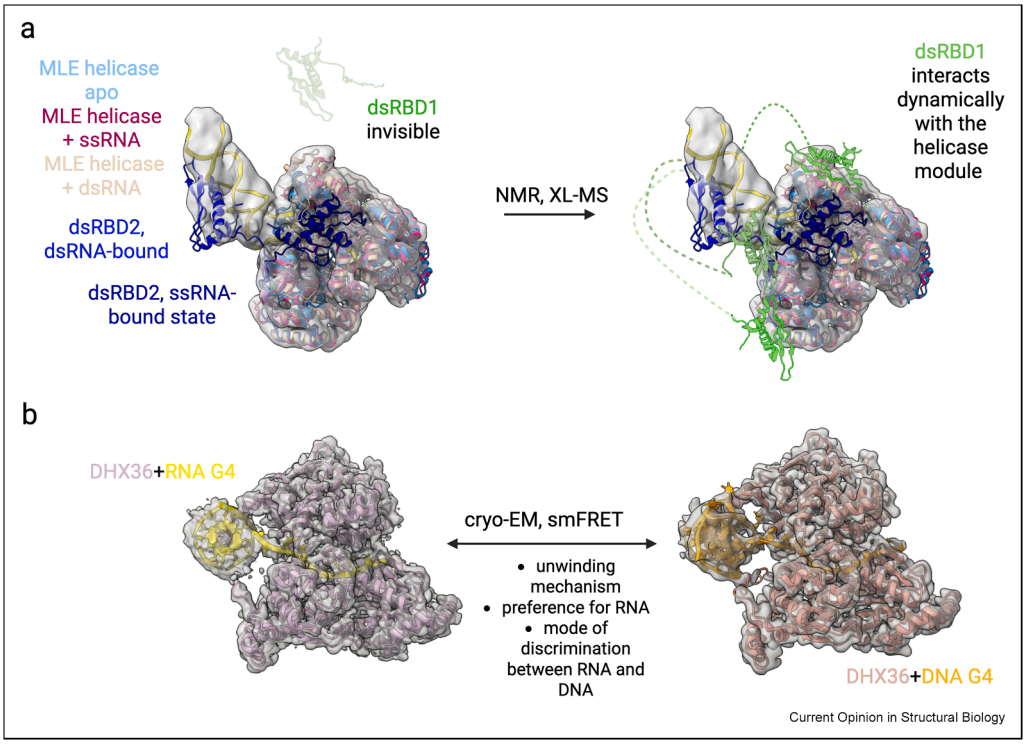
For example, single-molecule Förster resonance energy transfer (smFRET) was used to reconcile and place different cryo-EM snapshots of collided, coupled, and uncoupled bacterial expressomes onto a reaction trajectory, revealing how RNA polymerase and the ribosome cooperate in bacteria to optimize gene expression [47]. Using smFRET combined with NMR spectroscopy also showed how multivalent binding, RBP regions beyond canonical RBDs and synergies between RBPs and RNA remodeling can contribute to translation regulation [48,49].

The integration of NMR or smFRET with X-ray crystallography and cryo-EM has been particularly useful for understanding the enzymatic mechanism of RNA helicases. While cryo-EM could produce six different snapshots of the helicase maleless (MLE) during remodeling of roX2 lncRNA, explaining how the N-terminal dsRBD2 domain autoregulates the helicase function, NMR and XL-MS revealed that dsRBD1 interacts transiently with the helicase core (Figure 3a) [50]. Similarly, smFRET provided essential time-dependent data on DHX36 to complement structural snapshots, revealing the mechanisms of DNA and RNA G-quadruplex unfolding [51,52] and DNA vs. RNA substrate discrimination and the recognition of G-quadruplexes (Figure 3b) [53].

Integrative analyses also combine cryo-EM ensembles with live-cell imaging: A cryo-EM dataset comprising several snapshots of elongating ribosomes in different states [54] was used to model elongation dynamics and ribosome cooperativity in HEK cells [55].

Single-particle motility assays combined with NMR and SAXS have been used to propose a new model for the autoinhibition of *Drosophila* Kinesin-1 in complex with an RNA-adapter protein and RNA cargo [56]. Despite being studied for more than 40 years, our structural knowledge of the plus-end directed microtubule motor has been limited to its motor domain, with its mechanochemical cycle extensively characterized at high resolution (reviewed in Ref. [57]). However, the full-length motor, containing a long coiled-coil stalk and a

Figure 3



Integrative structural biology for helicases. (a) While cryo-EM snapshots of different states of the RNA helicase MLE could reveal the autoregulatory action and movement of dsRBD2, NMR and crosslinking MS (XL-MS) showed that the dsRBD1 of the helicase MLE, invisible in all six solved cryo-EM structures, interacts transiently and non-specifically with MLE's helicase module (PDBs 8PJJ, 8PJB and 8B9L) [50]. **(b)** Cryo-EM structures show versatile recognition of both RNA and DNA G-quadruplexes of different sizes by the helicase DHX36. Together with smFRET experiments, the modes of discrimination and RNA preference were revealed (PDBs 8VV2 and 8VX1) [53]. Created in https://BioRender.com. cryo-EM, cryo-electron microscopy; NMR, nuclear magnetic resonance; MS, mass spectrometry; smFRET, single-molecule FRET.

disordered tail domain, is a very flexible molecular complex, for which integrative methods to date seem imperative.

Condensate structural biology

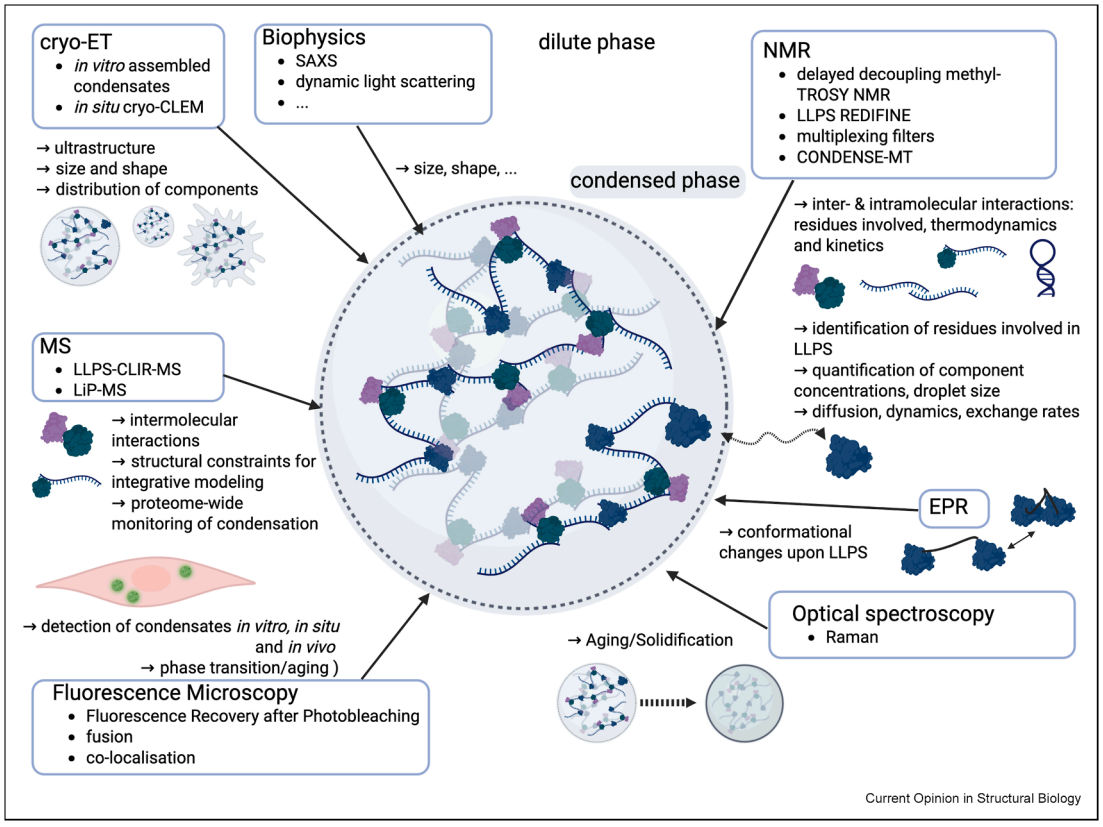
In recent years, the significance of biomolecular condensates formed by liquid-liquid phase separation (LLPS) in RNA biology has become increasingly evident. Membraneless compartments arise during many stages of RNA metabolism, including processing, transport and translational control, and their misregulation can contribute to disease [58,59].

Condensates formed by LLPS pose additional challenges for structural biology, as they are intrinsically disordered, dynamic and formed by multivalent interactions. For example, cryo-ET of *in-vitro*-formed condensates of *oskar* mRNA with its translational regulators Bruno or Hrp48 showed an amorphous state, in which little molecular detail is discernible [60]. *In situ* cryo-correlative light and electron microscopy (CLEM) recently showed stress granules in Hela cells as irregularly shaped, homogeneous ultrastructures with randomly distributed ribosomal components and no readily discernible scaffolds or cores at the given resolution [61].

Condensate structural biology thus calls for new methods (Figure 4) that can characterize LLPS-derived dense phases. Solution methods such as NMR and EPR are ideally suited to probe condensate properties in biologically relevant states, allowing the identification of residues involved in LLPS, the roles of protein-protein, protein-RNA interactions, and RNA−RNA interactions [62] and the quantification of component concentrations within droplets (reviewed in Ref. [63]).

In a recent example, NMR combined with biochemical and phase-separation assays revealed molecular mechanisms underlying condensate formation during *Drosophila* siRNA biogenesis [64]. Methyl-TROSY NMR of 2′-O-methyl labeled RNA was used to determine the thermodynamics and kinetics of RNA-RNA base pairing in condensates formed by the IDR of CAPRIN1, an RBP implicated in RNA transport, stability and translation [65,66]. Another study combined NMR and Raman

Figure 4



Structural biology of protein-RNA condensates. Methods and specialized experiments used to study biomolecular condensates are listed together with the information that can be obtained from them. Created in https://BioRender.com.

spectroscopic analysis of Fused in Sarcoma (FUS) condensates and revealed that aging droplets form β-sheet structures at their surfaces, providing molecular insight into the transition from dynamic liquid phases to more solid-like states [67].

A diffusion-optimized NMR spectroscopy-based pipeline, called LLPS REDIFINE, exploits the restricted diffusion of biomolecules inside the droplets and exchange dynamics between molecules in the condensed and dispersed phases to probe physical properties, such as diffusion constants, partitioning, droplet radius, and exchange rates, of the condensates by numerical analysis of the NMR data [68]. Multiplexed, label-free NMR enables simultaneous structural and dynamic analysis of multiple proteins inside biomolecular condensates [69].

A complimentary approach to study protein-RNA interactions inside biomolecular condensates is through cross-linking of isotope-labeled RNA and tandem mass spectrometry (LLPS-CLIR-MS) [70]. LLPS-CLIR-MS enables the characterization of intermolecular interactions present within as compared to outside biomolecular condensates at residue-specific resolution and the derivation of structural information on protein-RNA complexes within biomolecular condensates for integrative structural modeling of phase-separated RNPs. In-cell limited-proteolysis-coupled mass spectrometry (LiP-MS) can capture structural alterations upon condensate formation on a proteome-wide scale [71].

Collectively, these studies illustrate how integrative approaches combining solution spectroscopy, biophysical measurements, and MS can begin to reveal the molecular organization and dynamics of protein-RNA condensates, a class of assemblies that remains largely inaccessible to classical structural biology.

Summary and future challenges

Protein-RNA complexes are highly dynamic and heterogeneous, making them difficult to model with any single technique. Integrative modelling is thus inevitable for a complete characterization of these complexes and remains challenging, as each RNP requires tailored integrative approaches. Advances in AI, single-molecule techniques, and in-cell imaging now enable the integration of temporal and spatial information, moving the

field toward 4D structural biology [72,73]. Future efforts will focus on capturing the dynamic formation, rearrangement, and function of RNP assemblies in their native cellular and condensate environments, paving the way for predictive, time-resolved models of RNA metabolism and regulation.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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3738.

Data availability

No data was used for the research described in the article.

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