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Review Article

Protein–RNA interactions: from mass spectrometry to drug discovery

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Proteins and RNAs are fundamental parts of biological systems, and their interactions affect many essential cellular processes. Therefore, it is crucial to understand at a molecular and at a systems level how proteins and RNAs form complexes and mutually affect their functions. In the present mini-review, we will first provide an overview of different mass spectrometry (MS)-based methods to study the RNA-binding proteome (RBPome), most of which are based on photochemical cross-linking. As we will show, some of these methods are also able to provide higher-resolution information about binding sites, which are important for the structural characterisation of protein–RNA interactions. In addition, classical structural biology techniques such as nuclear magnetic resonance (NMR) spectroscopy and biophysical methods such as electron paramagnetic resonance (EPR) spectroscopy and fluorescence-based methods contribute to a detailed understanding of the interactions between these two classes of biomolecules. We will discuss the relevance of such interactions in the context of the formation of membrane-less organelles (MLOs) by liquid–liquid phase separation (LLPS) processes and their emerging importance as targets for drug discovery.

Introduction

Protein–RNA interactions are involved in many cellular processes, including, but not limited to RNA maturation, stability, translation, and host defence [1] (Figure 1). RNA-binding proteins (RBPs) play a crucial role in these fundamental cellular functions. Consequently, mutations that lead to aberrant RBPs or RBP-binding sites can have severe pathological implications. A typical eukaryotic cell contains tens to hundreds of thousands of protein and RNA species, whose dynamic interactions generate a huge number of transient and stable protein–RNA complexes. In the past, the number of existing RBPs has been significantly underestimated [1–3]. However, the recent improvements in RNA sequencing and mass spectrometry (MS) have facilitated the identification of RBPs from individual samples and up to the proteome-wide scale, defining the RNA-binding proteome (RBPome) [2,4].

Approaches to study the RBPome

Protein–RNA interactions can be studied from two angles, either by identifying proteins that are bound to a specific RNA or by identifying all RNAs that are bound to a specific protein. Nowadays, most methods are based on UV cross-linking (XL) of such ribonucleoproteins (RNPs) coupled to RNA sequencing or MS. Despite an increasing number of methodologies to study RBPs by MS, most follow a very similar, three-step strategy. In brief, the sample of interest is cross-linked by applying irradiation with UV light at 254 nm to covalently link RNA and protein at their contact site at virtually zero length distance. In a second step, the cross-linked molecules (RNA–protein conjugates or adducts) are enriched. This enrichment can

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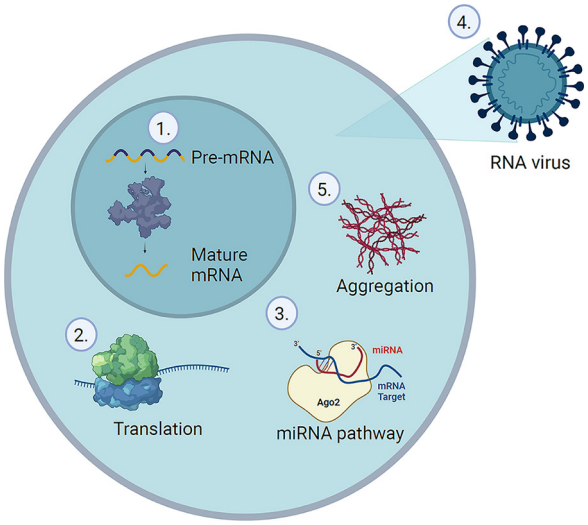

Figure 1. Examples for protein–RNA interactions in the cell

(1) Spliceosome, (2) ribosome, (3) miRNA-binding proteins, (4) RBPs related to viral replication, and (5) oligomers and aggregates of RBPs.

be achieved by many different means and will be discussed in more detail below. Finally, the enriched sample is further purified and cleaned for MS analysis.

In the last decade, many research groups have successfully developed protocols based on UV cross-linking MS to not only study single RNPs but also the RBPome of different organisms and cell types. Those methods can be divided into six main groups based on their enrichment strategy of RNA–protein or RNA–peptide conjugates (Figure 2):

1. The family of RNA interactome capture (RIC) protocols comprises a variety of more specialised workflows (vRIC [5], qRIC [6], eRIC [7], cRIC [8], RBDmap [9], serIC [10]) as well as peptide cross-linking and affinity purification (pCLAP [11]), which are all based on the enrichment of poly-adenylated RNA with oligo(dT) beads. This step is highly biased towards mRNAs, but allows stringent enrichment conditions due to the very specific and strong interaction between polyA tails and the beads.
2. RICK (RNA interactome using click chemistry) and CARIC (click chemistry-assisted RIC) utilise a non-native base, 5-ethynyluridine (5-EU) [12,13]. 5-EU is incorporated in newly transcribed RNA, modified by azide-biotin in a click chemistry reaction, and can be pulled down with streptavidin beads. Due to the high-affinity interaction between biotin and streptavidin, this approach also allows for stringent enrichment conditions.
3. RNA^{XL} further enriches for RNA–peptide conjugates with titanium dioxide solid-phase extraction after UV cross-linking and protein digestion with trypsin. Since the enrichment of RNA conjugates occurs at the peptide rather than the protein level, such a sample can identify the binding position of RNA and protein at single amino acid resolution as will be discussed in the next chapter [14].
4. The RBR-ID method does not include a dedicated enrichment step, but compares peptide intensities between cross-linked samples prepared with 4-thiouridine(4-SU)-labelled RNA and non-cross-linked samples. The mass shift introduced by covalently bound RNA will lead to a decrease in signal for successfully cross-linked peptides between the two samples [15].
5. 2C (silica-based solid-phase extraction), TRAPP (total RNA-associated protein purification), and RBS-ID utilise silica beads/membranes for nucleic acid enrichment [16,17].
6. The family of liquid–liquid phase extraction methods – orthogonal organic phase separation (OOPS), phenol–toluene extraction (PTex), and XRNAX (and recently developed derivatives, e.g., PPE, photoCAX, and targeted RNA–protein identification using OOPS (TROOPS)) – isolate protein–RNA conjugates based on their physiochemical properties [18–23]. Protein–RNA conjugates share properties of RNA and protein. During liquid–liquid phase extraction in an aqueous-organic (e.g., phenol/chloroform) system, cross-linked material will

form a layer in between the organic and aqueous phase. This interface layer can be extracted and further purified. A recently developed method that might be interesting, not from a systems-wide, but from a biochemical perspective is TROOPS. TROOPS is based on a previously published liquid–liquid phase extraction method to generate an intermediate, RBP-RNA-XL enriched sample that is used as input for a pulldown of a cross-linked RNA of interest [23].

Differences between existing protocols summarised above and methodological details of MS analysis are described elsewhere in detail [24–27], including tables with aggregated information about the above-mentioned methods [26,27].

Although UV-XL-MS is currently the gold standard to study RBPomes, it has some major disadvantages to consider, including low cross-linking efficiency (estimated at $<5\%$) and differences in reactivity depending on protein and nucleotide sequences, the relative orientation of amino acid side chains and nucleobases at the interaction site [28,29]. To overcome the low cross-linking efficiency of native RNA, a range of chemically modified bases, most prominently 4-SU, have been introduced; they are more photoreactive and significantly boost cross-linking efficiency. Another strategy that tries to overcome nucleobase preferences and the potentially low sample penetration of UV light uses chemical cross-linking reagents. These are often bifunctional chemicals that react with peptides on the one end and RNA on the other end. A comprehensive list of chemical cross-linkers can be found in the review by Fabris and co-workers [30].

From RBPome to structural read-out

In the past decade, the characterisation of RBPomes deriving from different samples and species has led to many insights into the world of RNA biology. RBPome-wide studies have catalogued an inventory of hundreds to thousands of RBPs in different species (4300 RBPs in human) (https://rbpbase.shiny.embl.de/) [2,4]. These data enable the collection of broad information about RBPs, frequently revealing insights into their structural organisation, like for instance the lack of canonical RNA-binding domains (RBDs) and the binding of RNA to unstructured regions in proteins [9]. Apart from that, many proteins that have been identified in RBPome-wide studies do not have a function known to be involved in RNA biology. Beckmann et al. named this group of RBPs 'enigmRBPs' [4], which includes many metabolic enzymes, particularly in the glycolysis pathway [31,32].

Although protein abundance is an important parameter in the cellular context, not only protein quantity but also protein localisation, post-translational modifications (PTMs) and conformational changes are vital for correct protein function. In the case of RBPs, binding to their target RNA and associated changes in, e.g., protein conformation add an additional layer of information that needs to be disentangled in order to understand a protein's function or an observed phenotype.

Multiple high-throughput approaches that utilise UV cross-linking to characterise the interaction between RNA and protein molecules down to single amino acid resolution have been described in the literature. RNA-sequencing-based approaches focus on extracting bound RNA from RNPs and perform sequencing on the recovered RNA. Regions that bind to the protein(s) will show reduced coverage by sequencing due to covalently linked amino acids at the binding site. Such differences in RNA coverage can be used for RNA footprinting, but require access to sequencing infrastructure. Trendel et al. proposed another approach that can be directly applied to MS-generated datasets. Their method takes advantage of the open modification search (OMS) strategy, which can identify virtually any peptide modification that results in a mass shift. An example for a widely used OMS software is MSFragger [33]. By treating RNA residues as a peptide PTM, OMS does not only identify whether a peptide is cross-linked to RNA but it also proposes the RNA composition (although not necessarily the correct nucleotide order). Additional postprocessing tools such as PTM-Shepherd can in some cases narrow down the binding site to a single amino acid [34]. Similarly, RBS-ID can precisely identify the RNA-binding sites at a single amino acid resolution by performing a complete digestion of the cross-linked RNA using hydrofluoric acid. This strategy focuses on uridine as the most commonly cross-linked base and has identified almost 2000 binding sites at single amino acid resolution with conventional software ('closed search') [35].

Among the specific software tools for RNA cross-linking data, Urlaub and co-workers developed RNP^{XL} [14]. For the identification of cross-linking sites, it is necessary to enrich peptide–RNA conjugates rather than protein–RNA conjugates. Metal oxides such as titanium dioxide bind compounds containing phosphate groups (e.g., on the RNA backbone or on phosphorylated amino acid) in a pH-dependent manner [36]. A similar strategy is employed by iTRAPP, which relies on a two-step enrichment protocol, TRAPP and TiO_2, and the cross-linking search engine called xiSEARCH [16]. RBDmap is a modified version of RIC that applies sequential digestion and polyA-tail enrichment of the cross-linked protein–RNA complexes to determine the region of the protein that is involved in RNA binding

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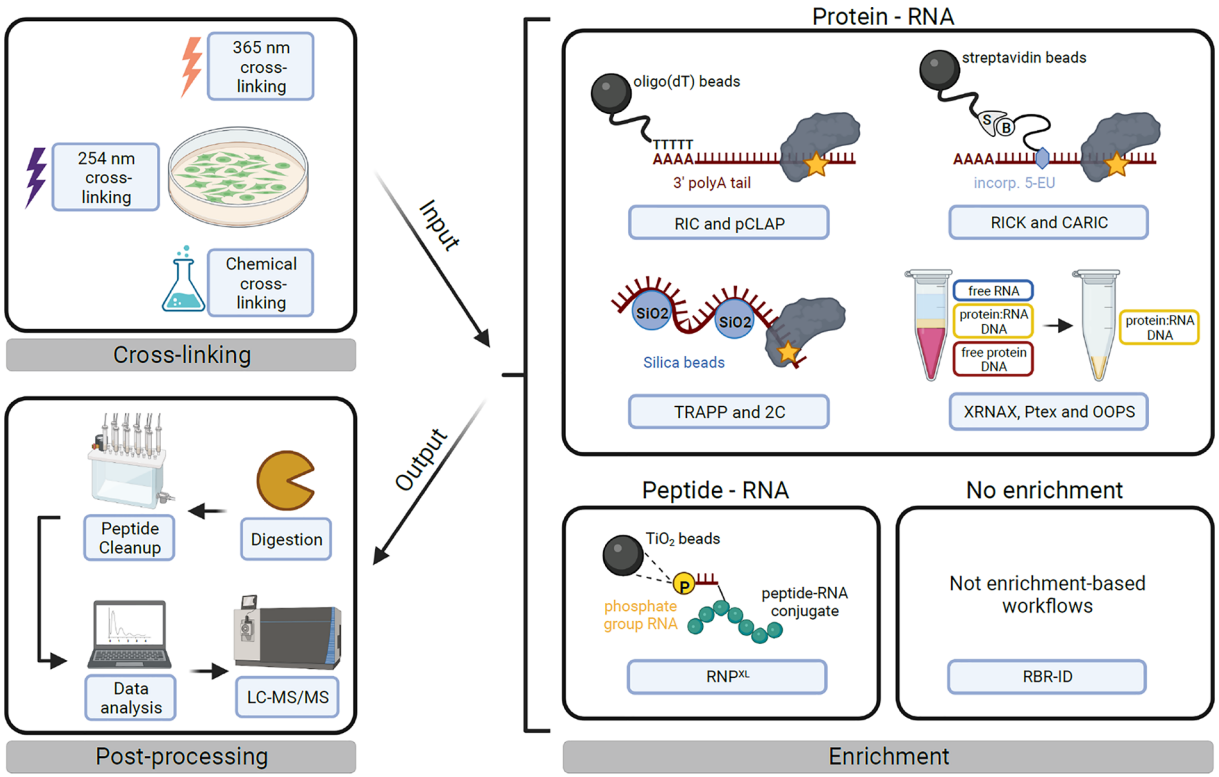

Figure 2. The protein–RNA UV cross-linking workflow

Schematic overview of the three steps – cross-linking, enrichment, and postprocessing – of an RBPome-wide UV cross-linking MS experiment. Cross-linking: RNPs can be cross-linked directly in cells (as depicted) using UV light, most commonly at 254 nm for native RNA, at 365 nm for 4-SU modified RNA or – less commonly – using chemicals. Note that cross-linking may also be performed in solution on lysates or purified complexes. Enrichment: this step is highly variable between protocols and can be split into six different modes of enrichment as described in the text. An alternative classification of protocols may be based on the input and/or output material for enrichment, which can be either protein–RNA (cross-links are represented as yellow stars) or peptide–RNA conjugates (as highlighted above the boxes). Although helpful, such a classification is practically more difficult to make, as RNA and protein digestion steps can be sequential or uncoupled. Postprocessing: postprocessing of enriched conjugates includes digestion with proteases and RNases (this step is dependent on the protocol used), clean-up of the digestion products, data acquisition by liquid chromatography tandem mass spectrometry (LC-MS/MS) and data analysis. Data analysis may be performed with conventional MS software or specialised XL-MS software tools (e.g., RNP^{XL}) [14].

[9]. Binding positions at a peptide level are also defined by pCLAP and RBR-ID methods [11,37]. Although these studies only identified binding regions in the small fraction of RBPs that are known today, they highlight the impact of UV cross-linking MS on structural biology, as in some cases 50% of the binding sites were found in intrinsically disordered regions (IDRs), which are difficult to model by conventional structural methods (discussed further below) [9].

A dedicated workflow that allows the characterisation of RNA–protein interactions of individual complexes or complex subunits is CLIR-MS (cross-linking of isotope-labelled RNA coupled to MS) [38]. Similar to RNP^{XL}, CLIR-MS is based on the enrichment of peptide–RNA conjugates with metal oxides and allows the identification of RNA–protein-binding interfaces down to the single amino acid level. In addition, incorporation of stable isotopes at specific positions or regions of the RNA sequence allows the determination of the binding sites on the RNA. Thus, CLIR-MS is very well suited for follow-up studies of proposed RBPs without additional structural information, if recombinant protein is available.

CLIR-MS has initially been used to pinpoint interaction sites between PTBP1 and one of its natural RNA targets, a part of the internal ribosomal entry site of encephalomyocarditis virus [38]. Recent applications include interactions between the SARS-CoV-2 nucleocapsid protein and s2m element, part of the viral RNA genome (discussed below) [39], and the RNA-binding properties of the ubiquitin-like domain of SF3A1 [40].

Studying the RBPome in a disease context

Based on their involvement in cellular housekeeping processes, mutated RBPs might be expected to cause system-wide effects. However, changes in structure and function of RBPs can also induce tissue-specific effects, which make them interesting (tissue-specific) therapeutic targets for drug discovery [2].

So far, few RBPome-wide studies have addressed the impact of tumorigenesis on the RBPome or *vice versa*. Mestre-Farràs et al. performed RIC experiments on both non-cancerous and metastatic cell lines and found that the RBPome significantly changes its RNA-binding activity in cancerous cell lines [41]. Specifically, the authors showed that RNA binding is required in the case of PDIA6, an ER-lumen chaperone, for its tumorigenic properties. Interestingly, PDIA6 was found to bind to RNA via a C-terminally located IDR. Other studies have exploited available RBPome datasets partially generated by UV cross-linking MS to find cancer-related RBPs or biomarkers for multiple cancer types by large-scale bioinformatic analyses [42–44]. Although these studies only make use of the experimentally determined RBP annotations, they highlight the potential of RBPome data for hypothesis generation and target selection for further functional studies.

The effect of viral infections on the host RBPome has also been investigated in a series of studies [5,8,45]. Modified protocols of RIC (cRIC and vRIC) were applied to show that approximately 25% and 33% of the host cell RBPome undergo remodelling upon infection with SINV or SARS-CoV-2, respectively. Comparison of the RNA interactomes between viruses and host cells revealed a broad overlap of 60%, suggesting that different viruses may share common host RBP targets [5,8]. Additionally, Kim et al. developed VIR-CLASP, another method to study RBPome interactions between viral RNPs and the host cell [45].

Future large-scale RNA interactome screens may thus offer candidate RBPs as new therapeutic targets in the oncology and infectious disease areas, among others. However, applications towards drug discovery require detailed structural characterisation of target proteins and protein–RNA complexes. This cannot be achieved by MS alone, but requires input from structural biology and biophysics techniques, which will be discussed in the following.

Structural biology and biophysics of RBPs

The majority of RBPs possess a modular architecture, with the key components being globular RBDs, such as RNA recognition motif, K homology domain, zinc finger, and others [46,47]. Each RBD is characterised by its distinct specificity and affinity in the RNA-binding process [48]. As some RBD types are characterised by a low RNA sequence specificity (*vide* cold-shock domain [49]), they often co-occur (along with high specificity domains) in order to enhance the specificity and affinity of RNA binding [46]. These domains are flanked and connected by linkers that are often characterised by a high degree of conformational flexibility, and thus referred to as IDRs. Longer linkers allow the independent recognition of more than one RNA motif by the connected domains, while shorter linkers can mediate joint RNA binding across domains [46,48]. However, in recent years, it has become clear that some linkers may be directly involved in RNA interactions on their own [50–52]. Upon RNA binding, the disordered linker may adapt its structure via induced folding, called disorder-to-order transition [52]. This can result in a rearrangement and proper positioning of two RBDs flanked by transitioning linkers and thus the formation of a larger RNA interaction surface of a protein [53].

IDRs show poor RNA sequence specificity, and their interaction is thought to be electrostatically driven [54], as they often consist of hydrophilic residues and carry a large net charge [52]. Frequently, they also feature repeats of arginine/serine (RS repeats), arginine/glycine (RG repeats), short linear motifs, low complexity sequences, and electronegative clusters [46,52,55]. These distinctive patterns reduce the informational content of the linker sequence; however, they do play a role in the variety of biochemical and physiological processes, such as RNA metabolism, binding, folding, and transportation [52,56]. IDRs are also rich in PTM sites and are therefore essential for the regulation and control of many cellular processes. In RBPs, arginine (methylation), tyrosine, and serine (phosphorylation) are the most frequently modified amino acids. For example, it was shown that methylation of DDX4 and FUS/TLS RBPs reduced the formation of membrane-less organelles (MLOs) by these proteins [57,58]. Moreover, long homorepeats of single amino acids may be present, such as poly-A or poly-G, poly-K/R or poly-N/Q [59]. While being involved in many physiological events, both low complexity domains (LCDs) and long homorepeats have also been found to facilitate liquid–liquid phase separation (LLPS) events [48,52,59,60]. As a result, MLOs are formed as higher-order assemblies of RBPs and RNA due to the coacervation of protein–RNA assemblies from the surrounding solvent [61].

These large biomolecular condensates (Figure 3) can be characterised as thermodynamically reversible, highly dynamic, and liquid-like, as they are prone to dissociate upon increase in temperature or salt concentration, pH changes, and can undergo fusion and shear force deformation [54,62]. The membrane-less architecture of liquid compartments that are separated from the cyto- or nucleoplasm allows a rapid and reversible exchange of their content with the

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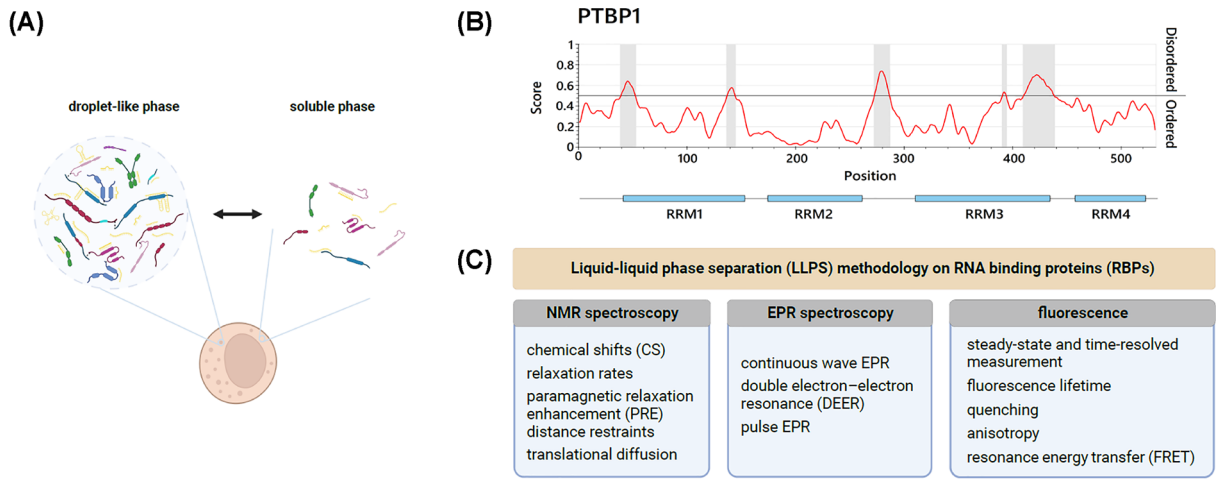

Figure 3. Liquid-liquid phase separation of RNA-binding proteins

(**A**) Schematic representation of coexisting, reversible droplet-like (condensed), and soluble (dispersed) phases in the cytoplasm. (**B**) Prediction of disordered protein regions of the RBP PTBP1. Identified regions (highlighted in grey) are mainly located in flexible linkers, flanking RBDs. Generated using IUPred3 [65]. (**C**) A summary of commonly exploited methods to study LLPS phenomena involving RBPs *in vitro*.

plasma surrounding and provides a distinct microenvironment and physical constraints [48,54]. Typical MLOs consisting of RBPs and RNA include cytoplasmic processing bodies (P-bodies, PBs), stress granules (SGs), and P-granules, but also nucleoplasmic Cajal bodies, nucleoli, nuclear speckles, and paraspeckles [48,52]. MLOs are involved in a myriad of cellular processes, ranging from protein synthesis, RNA metabolism, transportation, and regulation to stress response and cell signalling [52,63]. They form rapidly under stress conditions (temperature, pH, starvation) as a response to environmental stimuli. Furthermore, MLOs were shown *in vitro* to undergo a spontaneous transition to more solid-like structures, such as hydrogels and fibrils [54,64].

The mechanisms of formation of liquid droplets remain largely unknown. So far, the majority of studies predominantly put an emphasis on the role of proteins in LLPS, neglecting the RNA contribution. As it was previously mentioned, amino acid sequence motifs were shown to govern the coacervation of assemblies, for example, tyrosines and arginines in FUS [57] or aromatic residues in hnRNPA1 [66]; unspecific hydrophobic, electrostatic, and hydrogen-bonding interactions were also found to contribute [66,67]. Interestingly, glycine-rich motifs enhance fluidity of liquid droplets while glutamine and serine residues promote their hardening [57]. On the other hand, RNA itself was found to undergo LLPS on its own and serves as a nucleation core in the formation of YB-1-rich SGs.

Biophysical properties of coacervated structures largely depend on RNA characteristics such as length, sequence, and secondary structure [68]. So far, LLPS has been studied *in vitro* on several RBPs, such as DDX4, hnRNPA2, CAPRIN1, FUS, TDP-43, TIA-1 [52,66], DHH1 [69], PTBP1 [70], SARS-CoV-2 nucleocapsid protein [67], and many others. As the concept of LLPS gained widespread attention over the last few years, six databases collecting the reports of proteins and RNAs undergoing phase separation have been established, namely LLPSDB, PhaSePro, PhaSepDB, DrLLPS, RNAGranuleDB, and RNAPhaSep [68].

Because of the dynamic and conformationally flexible behaviour of LLPS assemblies, the proper choice of research methods for characterising them remains non-trivial. In *in vitro* studies, nuclear magnetic resonance (NMR) spectroscopy has taken the lead as it provides detailed information on structure, transient interactions, and molecular motions across different timescales, even of highly dynamic macromolecular regions [61]. In comparison, other structural biology techniques like cryoelectron microscopy and X-ray crystallography have the disadvantage of providing only a static picture of analysed molecules and a poor insight into disordered regions of proteins [69]. Notably, NMR-based experiments elucidated the importance of unspecific and transient interactions between proteins and RNAs (for FUS, DDX4, hnRNPA1), the role of specific interactions and dimerisation (for TDP-43) and PTMs (for TDP-43, FUS family proteins, hnRNPA2) in liquid droplet formation [57,60,61,66]. NMR methods are, however, limited by protein size, show increased redundancy due to the presence of tandem repeats and provide little insight into the overall shape and mobility of LLPS droplets [71]. Electron paramagnetic resonance (EPR) spectroscopy is,

in contrast, not limited by these factors. So far, it has been successfully used to determine the intramolecular distance distribution in liquid droplets of FUS [72] and the role of oligomerisation in phase separation of TDP-43 [73]. EPR-derived detailed structural information requires cryogenic temperatures; however, this raises concerns about possible structural changes of formed droplets and their macromolecular components [66].

Various fluorescence techniques, such as anisotropy, quenching, Förster resonance energy transfer (FRET) or steady-state and time-resolved measurements, have been widely utilised to provide insight into many LLPS features, like formation, dynamics, and compactness of FUS and TDP-43 droplets [57,60,74]. Altogether, these spectroscopic techniques provide complementary information about protein–RNA interactions, especially for dynamic and often transient LLPS systems, contributing to further exploitation of these interactions and their manipulation in biological systems.

In addition, MS-based methods can deliver information on interactions within droplet-like condensates, taking into account those driven by IDRs as well. Cross-linking MS of protein–protein interactions has been successfully exploited so far in a few cases of, e.g., FUS and SARS-CoV-2 nucleocapsid protein [75–77], while applications of protein–RNA cross-linking in connection with LLPS have not yet been reported in the literature.

RBPs and drug discovery

Drug development relies on detecting compounds that can specifically bind to target molecules and may be potential candidates for development as a medical treatment. Benchmark techniques to study molecules that may also inhibit or interfere with protein–RNA interactions specifically include NMR-based screening, X-ray crystallography, and structural MS. In addition, computational techniques have been proven to be an excellent tool, reducing the time and cost of drug discovery by predicting the interactions between a protein and a ligand. Finding the suitable ligand is based on a virtual screening of molecular libraries, which contain data obtained by the above-mentioned traditional techniques. High-throughput screening is performed based on algorithms that use protein properties and binding site information to select the suitable molecules with desired protein specificity and affinity [78,79]. In addition to this, molecular-docking simulations allow protein–ligand interaction predictions at the structural and thermodynamic level [80]. Li et al. moved one step further by introducing MONN, a deep-learning tool, which explores the mechanisms behind protein–ligand interactions and predicts the binding sites between the two molecules [81]. However, despite the recent advances in bioinformatics, certain features of interactions still need to be further explored in order to obtain reliable computational data, such as including flexible protein regions in high-throughput docking or including protein–ligand-binding kinetic rate calculations [82]. Combining experimental data, including cross-linking data in the form of distance restraints, might be a particularly promising direction to overcome the deficiencies of purely computational methods. This was recently shown for the SARS-CoV-2 nucleocapsid protein in complex with the viral RNA element, s2m [39] (Figure 4).

As mentioned above, RBPs regulate various biological functions of RNA and *vice versa*; not only in physiological but also in disease-affected pathological conditions, and can be related to many human diseases, such as cancer, autoimmune diseases, neurodegenerative and cardiovascular diseases, as well as viral infection and virus pathogenesis [83–88]. Consequently, RBPs could be potential therapeutic targets for drug development [89]. However, RNPs are conformationally highly dynamic systems, which pose many challenges for drug discovery. Strategies to approach this assignment could be developed based on three objectives:

- Drugs that directly target protein–RNA interactions. These could include compounds that react either with the protein or with the RNA molecule by accessing their binding sites and blocking protein–RNA interactions [89–91], as exemplified by recent work on the N protein of SARS-CoV-2 (Figure 4).
- Drugs that render the protein or the RNA dysfunctional. N protein, for instance, plays a key role in the life cycle of the virus and therefore inducing N aggregation could inhibit viral replication [92,93].
- Drugs that can decrease the levels of pathological protein aggregates as in the case of neurodegenerative diseases [84].

Furthermore, the exact mechanism on which the drug's action is based on should be fully understood and it should ideally remain an irreversible process. Cell permeability, efficient drug delivery, and tissue distribution are some drug characteristics that should be taken into consideration when a drug is designed [84,94,95]. Additionally, given that delivering the drug to the desired organ or tissue can be challenging, the use of artificial intelligence and computer modelling seems to be a way to address these issues [96]. All of the factors are particularly challenging when targeting the RNA part of protein–RNA interactions.

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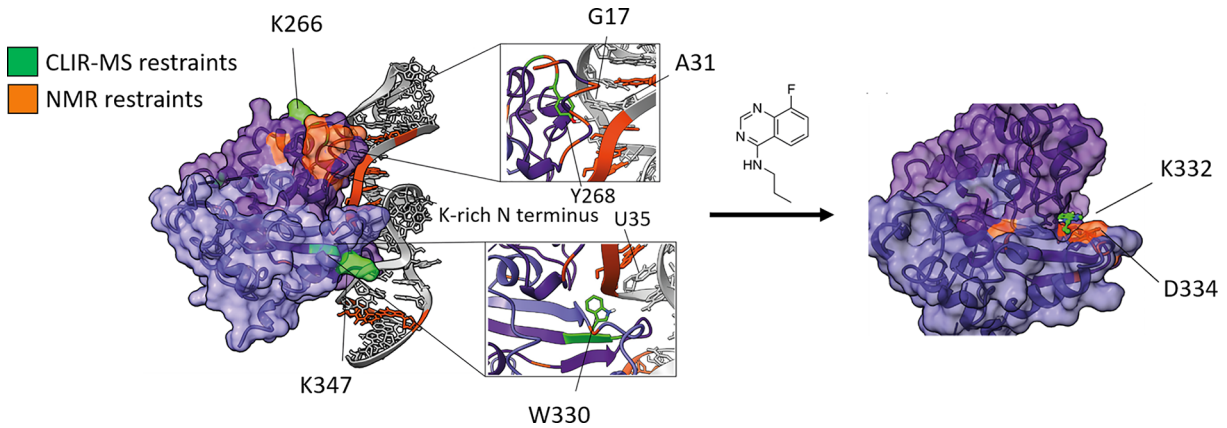

Figure 4. Fragment screening to find inhibitors for the interaction between SARS-CoV-2 nucleocapsid protein and the s2m element of the viral RNA

A computational model of the protein–RNA complex was generated based on information obtained from CLIR-MS and NMR spectroscopy (left), and small-molecule fragments were identified that bind to either the protein (shown here on the right) or the RNA part of the complex. Reproduced from [39].

Conclusions

As we have shown, a diverse set of experimental and computational tools exists to characterise the interactions between proteins and RNA. MS-based methods have seen an enormous growth in recent years, taking advantage of the high sensitivity of this method and its ability to deal with samples of high complexity. However, it is apparent that other methods will continue to play a crucial role to obtain a comprehensive picture of the protein–RNA interactome. We believe that the integration of different methods will soon provide us with a better understanding even of complex phenomena such as LLPS processes *in vitro* and *in vivo*. This should form the basis for further biological and biomedical discoveries, including new directions towards the treatment of diseases.

Summary

- Cross-linking methods in combination with MS detection have emerged as a key player to characterise individual protein–RNA complexes and the RBPome.
- MS-based methods are complementing structural biology and biophysical techniques to study protein–RNA interactions.
- An increased understanding of the role of protein–RNA interactions in health and disease suggests that an integration of MS- and non-MS-based techniques will provide crucial insights into fundamental biological processes such as LLPS and a new strategy for drug discovery target selection and characterisation.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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Abbreviations
4-SU, 4-thiouridine; 5-EU, 5-ethynyluridine; CARIC, click chemistry-assisted RNA interactome capture; CLIP, cross-linking and immunoprecipitation; CLIR-MS, cross-linking of isotope-labelled RNA coupled to mass spectrometry; EPR, electron paramagnetic resonance; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; FUS/TLS, fused in sarcoma/translocated in liposarcoma; IDR, intrinsically disordered region; LC-MS/MS, liquid chromatography tandem mass spectrometry; LLPS, liquid–liquid phase separation; MLO, membrane-less organelle; MS, mass spectrometry; NMR, nuclear magnetic resonance; N protein, nucleocapsid protein; OMS, open modification search; OOPS, orthogonal organic-phase separation; pCLAP, peptide cross-linking and affinity purification; PTM, post-translational modification; RBD, RNA-binding domain; RBP, RNA-binding protein; RBPome, RNA-binding proteome; RIC, RNA interactome capture; RICK, RNA interactome using click chemistry; RNP, ribonucleoprotein; SARS-CoV-2, severe acute respiratory syndrome coronavirus type 2; SINV , sindbis virus; TRAPP, total RNA-associated protein purification; TROOPS, targeted RNA–protein identification using OOPS.

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