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NMR of RNA - Structure and interactions

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Abstract

RNA was shown to have a more substantial role in the regulation of diverse cellular processes than anticipated until recently. Answers to questions what is the structure of specific RNAs, how structure changes to accommodate different functional roles, and how RNA senses other biomolecules and changes its fold upon interaction create a complete representation of RNA involved in cellular processes. Nuclear magnetic resonance (NMR) spectroscopy encompasses a collection of methods and approaches that offer insight into several structural aspects of RNAs. We review the most recent advances in the field of viral, long non-coding, regulatory, and four-stranded RNAs, with an emphasis on the detection of dynamic sub-states and in view of chemical modifications that expand RNA's function.

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Introduction

When the human genome project was introduced, a large part of the genome was identified as non-coding and hence considered redundant. It has, however, become clear that a large part of the genome is indeed transcribed into different flavors of regulatory RNAs. One must, therefore, factor in the structural elements of RNA, their interconversion, and interactions to understand and steer biological processes. Among different methods presenting atom-specific details of an RNA structure, nuclear magnetic resonance (NMR) is a method of choice for dynamic systems that might be problematic to study with either X-ray crystallography or cryoelectron microscopy. Different NMR methods exploit various NMR observables, such as chemical shift, through-space and through-bond interactions, and relaxation phenomena to provide an elaborate description of a macromolecular system under investigation (Figure 1).

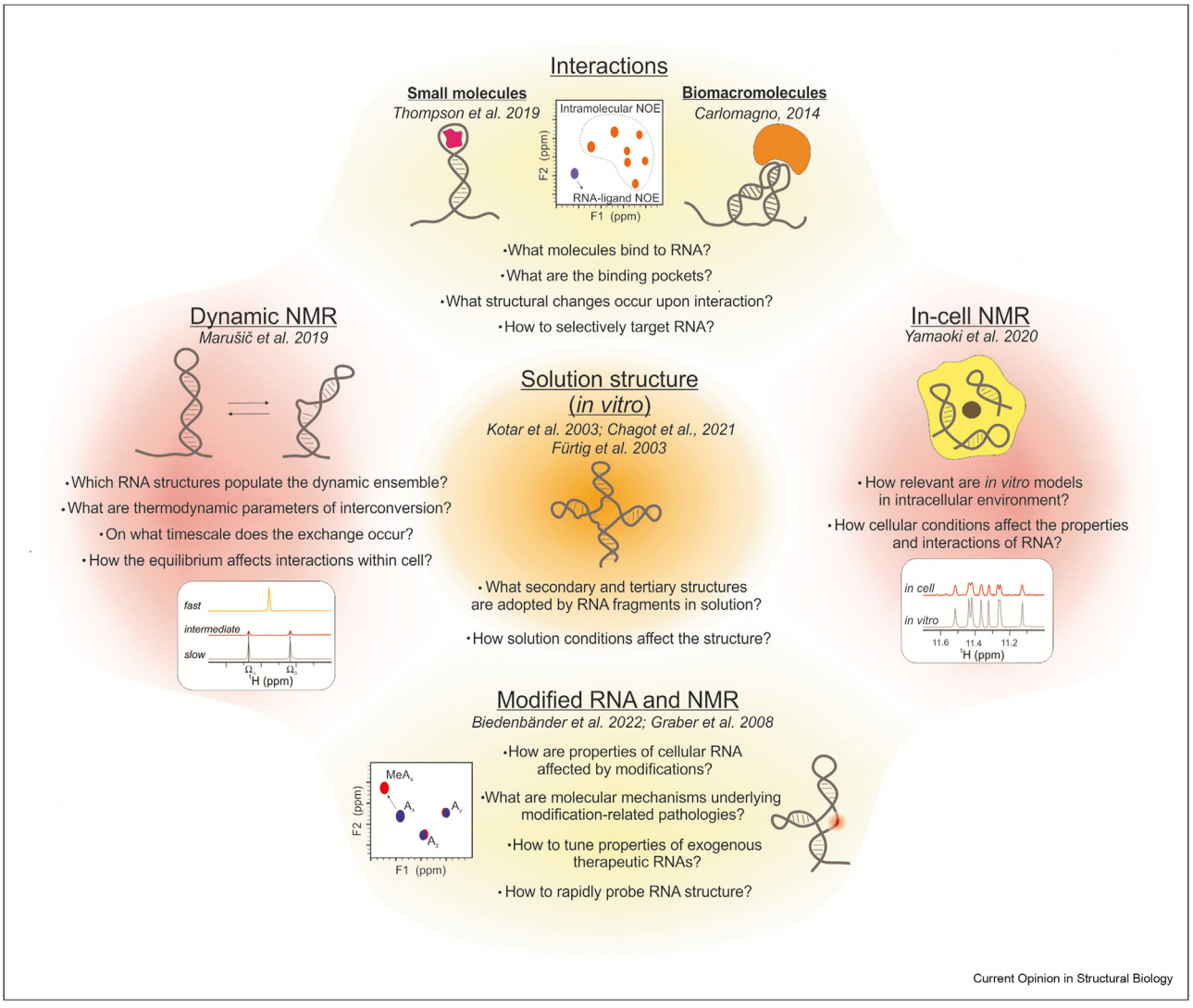
NMR analysis of RNA ranges from a simple and quick experimental setup with unlabeled RNA for sample screening to often lengthy procedures required for structure determination with labeled RNA samples (Figure 2). Fortunately, RNA resonances characteristic of hydrogen-bonded protons within base pairs are observed in an otherwise unpopulated part of proton NMR spectra, offering excellent fingerprint-like signatures for different RNAs. The signature of base-paired protons is, therefore, often used to guide the so-called divide and conquer approach, where a small fragment corresponding to a large RNA molecule is prepared in a way to maintain structure and enables a more detailed exploration (Figure 2, bottom). Secondary structure can be determined from proton NMR spectra in tandem with secondary structure prediction programs (Figure 2, middle). The main disadvantages of solution-state NMR are spectral overlap with increasing RNA size and high molecular weight limit resulting in so-called NMR invisible species. Different labeling schemes used in sample preparation with either solid-phase synthesis or enzymatic *in vitro* transcription are harnessed to reduce spectral overlap and use a set of through-bond NMR experiments necessary for 3D structure determination (Figure 2, top). On the other hand, large molecular weight RNA or RNA complex calls for the use of complementary methods, such as SAXS and SANS, or to transition to solid-state NMR.

In this review, we discuss the most recent topics in RNA structural biology with NMR as a method of choice. We also highlight the directions in which RNA structural research is headed: viral and other large RNAs, dynamic biological processes in which RNA stars as the main character, and chemical modifications that finetune RNA properties.

Structural diversity from viral RNA to long non-coding RNAs

The pandemic of COVID-19 resulted in the initiation and bottom-up organization of NMR and a wider

Figure 1



Challenges in RNA structural biology addressed by NMR [1–9]. Different types of biomolecular NMR experiments are often combined to provide insights into mechanisms involving RNA at the molecular level. RNA (secondary) structure determination *in vitro* usually precedes the study of other aspects, such as structure diversity and related kinetics of interconversion, intermolecular interactions, dynamic processes, and effects of nucleotide modifications both *in vitro* and in cellular conditions.

community of researchers in the COVID-19-NMR project aimed to structurally characterize proteins and especially nucleic acids involved in the regulation of SARS-CoV-2 replication and translation. Joined efforts of the community using NMR and complementary methods, such as dimethyl sulfate (DMS) footprinting and 3D modeling, have resulted in the characterization of 15 conserved RNA elements in the ribosomal frameshift segment and the 5′- and 3′-untranslated region of the Sars-CoV-2 genome [11]. An NMR-based screening of a fragment library identified RNA elements that are potential drug targets and enabled the identification of key functional units for binding in ligands [14]. Among others, amilorides were identified to bind 5′-UTR and adjacent SL domain of the SARS-CoV-2 genome with the effect of reducing viral replication [15].

Not just SARS-CoV-2 but also other viruses and especially those with their genetic material in RNA form are attractive systems for structural characterization with NMR. Stability [12] and interactions [16] of cis-acting regulatory RNA elements of hepatitis C and Dengue West Nile virus were shown to affect viral replicative fitness. For hepatitis B, a binding pocket of ε element was identified that draws viral polymerase to initiate reverse transcription and can serve as a target for small molecule interventions and thus complement current antiviral therapies [13].

Studies on viral genomes need to create smartly selected short fragments to be amenable for NMR characterization and at the same time reflect biological function. Very similar problems are faced in the field of regulatory long non-coding RNA (lncRNA) molecules, transcripts of more than 200 nucleotides (nt) that are not translated into protein, but are involved in gene regulation. They are attractive for NMR characterization due to their flexible character and structural elements involved in multivalent interactions. Namely, experimental determination and reliable computational prediction for large RNAs are extremely challenging. Several approaches to tackle this issue have recently been used. Ohyama et al. [10] have reported on the secondary structure determination of 167 nt SINE B2 element of Uchl1 lncRNA by systematically dividing the full-length SINE B2 into smaller units while making sure that their structures correspond to the original one. NMR signals in the imino region of 1D proton NMR spectra were used as indicators of conserved fold and compared to the secondary structure obtained by the secondary structure prediction program using restraints derived from SHAPE data. Interestingly, NMR has shown that one of the domains computationally predicted to form a stem structure does not form under *in vitro* conditions. Abzhanova et al. [17] estimated the conformational heterogeneity of all exonic regions of HOTAIR lncRNA with native gels at different $MgCl_2$ concentrations. Exon 4, which displayed no variability under any of the salt conditions, was selected for structural characterization by both NMR and SHAPE. This joined exon-biased and integrated biophysical approach presents a strategy to examine conformational heterogeneity in lncRNAs and emphasizes NMR as a key method to validate base-pair interactions in large RNAs to corroborate their structure.

Typical NMR observables are limited to short distances within 5 A, which is especially problematic for large RNAs. A fresh approach to tackle this problem was demonstrated by Strickland et al. A segment of the RNA of interest was replaced by another that contains the specific binding sequence for paramagnetically tagged U1A protein. The complexation of U1A with the chimeric RNA indirectly provided long-range (~50 A) structural information on the target RNA via the measurement of large chemical shift differences induced by the vicinity of the tag. The attractiveness of the approach is in the indirect tagging of the RNA molecules and the possibility to study very large—above 200 nt—RNAs [18].

Riboswitches are regulatory elements of mRNA that change conformation upon a small molecule binding and subsequently affect mRNA translation levels. Because of the dynamic nature of riboswitches, they are convenient for exploration with NMR methods. Recently, Mg^{2+} requirements and guanidinium binding site were explored for guanidine II riboswitch with the use of NMR and paramagnetic relaxation enhancements, while diffusion-ordered NMR was used to discern between the intramolecular kissing loop and dimer formation. An ACGR (R is any nucleotide) loop was identified as vital for dimerization and guanidine binding [19]. The structure of ZMP (5-aminoimidazole-4-carboxamide ribonucleotide) sensing riboswitch and its ability to bind ligand was found to be affected by transcript length, enabling the sensing of conditions in the bacteria to regulate gene expression [20].

Renewed interest in the heterogeneity of four-stranded RNA structures

RNA G-quadruplexes are secondary structures often present in UTR and coding regions of mRNA. While the role of G-rich mRNA regions in the regulation of transcription and post-transcriptional events was confirmed, the possibility of forming RNA G-quadruplexes inside the cell is often debated. A significant breakthrough was achieved by the group of Xu, which managed to show the formation of mono- and bimolecular telomeric RNA G-quadruplex in intracellular conditions, using methods of ^{19}F in-cell NMR [21].

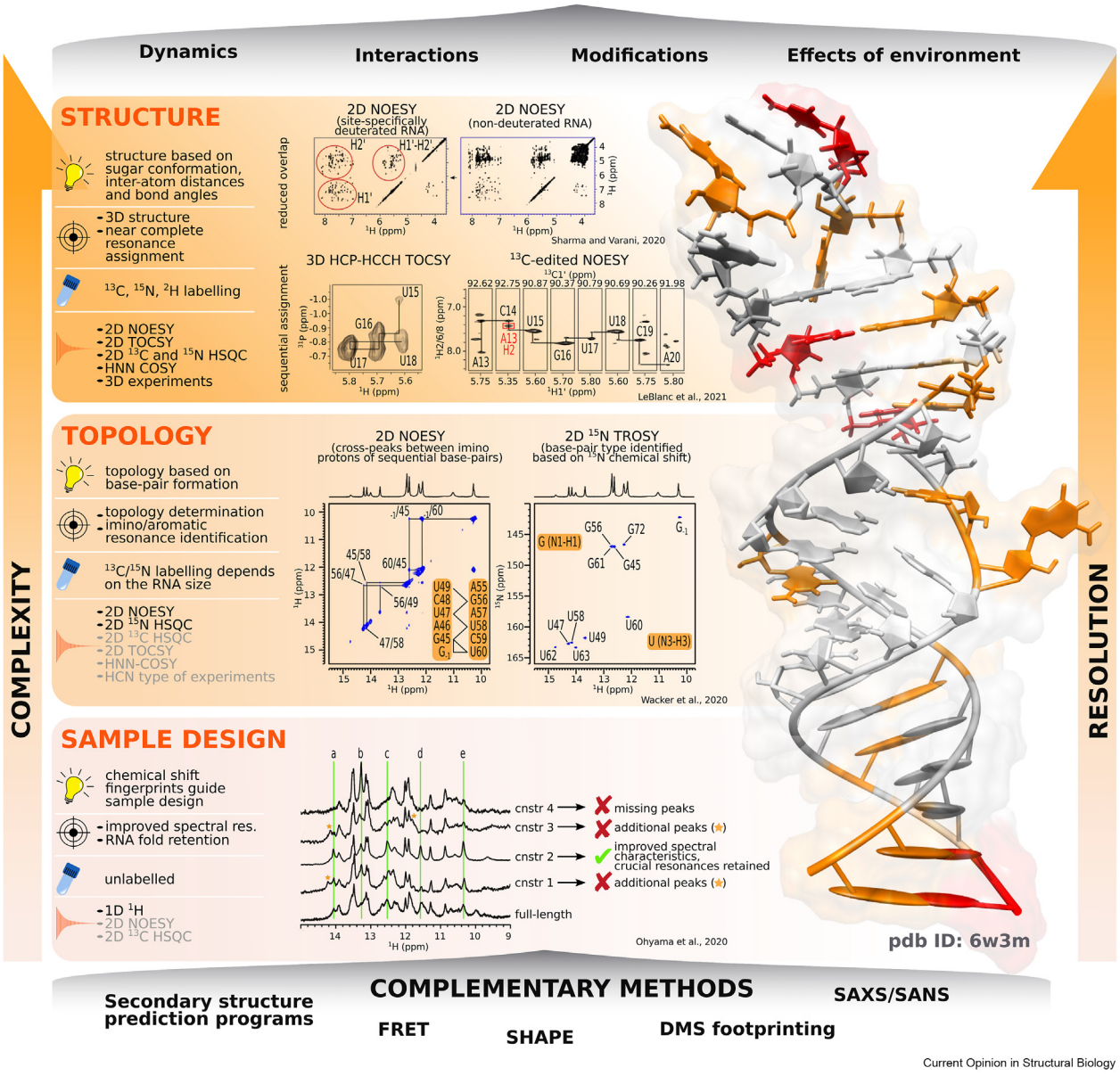
The important regulatory role of RNA G-quadruplexes makes them a potential drug target. Development of corresponding drugs requires a detailed understanding of G-quadruplex structural behavior. NMR methods have recently helped to reveal factors affecting the topological trends, stability, and dynamics of RNA G-quadruplexes comprising canonical and non-canonical structural elements [21—24]. An important insight into the differences in folding processes of human telomeric repeat-containing RNA and DNA was made by the Schwalbe group [25]. A set of kinetic data enabled them to suggest that RNA G-quadruplex folding occurs faster due to the absence of the *syn/anti*-conformation flips of nucleobases, which contrasts the behavior of DNA oligonucleotides.

The origin of the strong stabilization effect of reversed polarity 3′-terminal U-tetrad in G-quadruplex was attributed to the formation of a hydrogen bond between uridine ribose moiety and the phosphate group of preceding guanine residue by direct observation of hydrogen and phosphorous coupling via the hydrogen bond [22]. Moreover, naturally occurring modification of adenine to inosine residue results in the formation of G-quadruplexes with mixed guanine-inosine tetrad. Since adenine-to-inosine mutations tend to occur in cells, it shows the possibility for activation of latent G-quadruplexes, which may be a part of gene expression regulatory processes [24].

Dynamic features of RNA as regulatory elements in biological processes

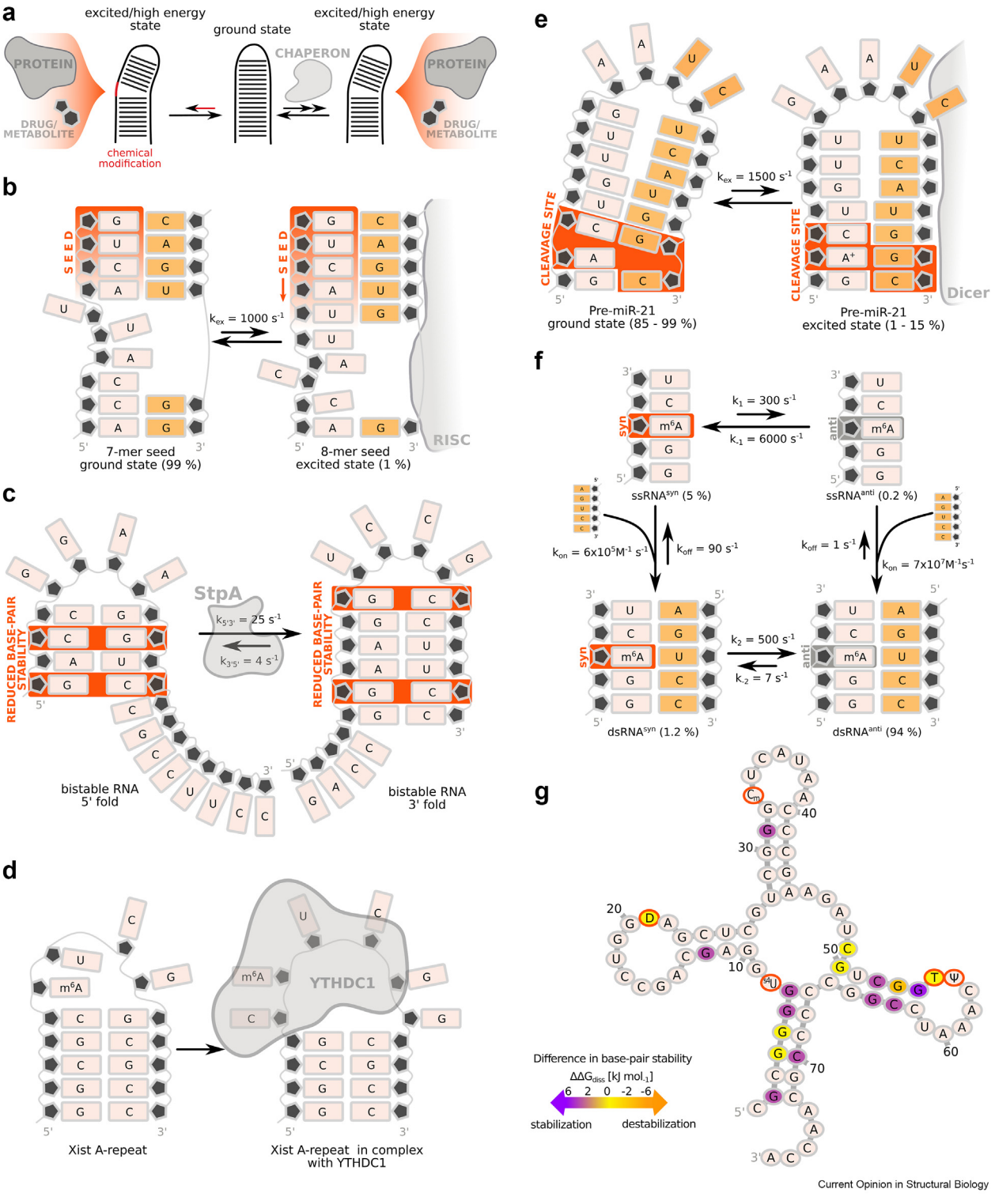
The study of RNA dynamics (Figures 1 and 3a) with NMR methods has been an area in which truly

Figure 2



Different characteristics of RNA molecules can be explored with diverse NMR experiments that offer varying degrees of information. The increase in resolution goes in parallel with the complexity of sample preparation and NMR experimental setup. Assignment of atoms in residues of interest is a necessary first step for all experiments that seek to answer questions not related solely to topology/structure determination related to dynamics, interaction, modification, and effects of the environment. **SAMPLE DESIGN**: initial sample selection is typically supplemented with information on topology, the overall shape, and binding surfaces reported by complementary methods. 1D proton spectra illustrate the selection of SINE B2 fragment based on full-length SINE2 lncRNA chemical shift fingerprint [10]. **TOPOLOGY**: base-pair formation results in characteristic cross-peaks in NMR spectra. The proximity of base-paired protons gives rise to connectivities in the NOESY spectrum that can be followed along the base-paired RNA stem. 2D spectra display topology determination of 5'-genomic end construct5 SL2+3 of SARS-CoV-2 genomic RNA [11]. **STRUCTURE**: Different labeling schemes are used to exploit through-bond NMR experiments and obtain inter-atom distance and angle restraints used in structure calculation. NMR spectra show the use of deuterated RNA sample to reduce the number of cross-peaks for stem-loop B of DENV4 virus [12] and through-bond NMR experiments used for sequential assignment of ε priming loop of hepatitis B virus [13]. NMR spectra are reprinted with permission from Refs. [10–13].

Figure 3



Dynamic events in RNA affect biological outcomes. (**a**) Schematic representation of dynamic RNA behavior with highly populated ground state in equilibrium with short-lived, high-energy state targeted by cellular proteins, metabolites, and (potential) drugs. Dynamic behavior can be induced or stabilized with the introduction of chemical modifications and modulated by chaperon proteins. (**b**) The excited state of the miR-34a – mRNA duplex is recognized and preferentially cleaved by RISC. (**c**) RNA chaperon StpA accelerates refolding of bistable RNA by destabilization of end base pairs. (**d**) Increased dynamics in stem loop of m^{6}A-modified Xist A-repeat increases accessibility for reader protein YTHDC1. (**e**) The excited state of Pre-miR-21 is recognized and preferentially cleaved by Dicer. (**f**) m^{6}A decreases the annealing rate and destabilizes double-stranded RNA via access to the excited

remarkable results have been obtained that managed to pinpoint the regulation mechanisms of several important biological processes. The power of dynamic studies with NMR has been recently shown for microRNAs that regulate diverse biological processes. Baronti et al. used NMR relaxation dispersion and mutagenesis to demonstrate that microRNA processing complex RISC selects and more efficiently cleaves the elongated stem of an excited state (Figure 3b) [26]. Baisden et al. have uncovered a pH-responsive excited state of microRNA-21 precursor that is formed on the reshuffling of the secondary structure of the entire apical stem-loop region and is more efficiently processed with Dicer (Figure 3e) [27]. In both cases, the excited state better represents substrate requirements and is, therefore, more efficiently cleaved by processing proteins.

The idea that excited states represent a hidden layer for regulation opens new strategies in RNA-targeted therapeutics and identifies a need to better recognize and design RNA conformational ensembles. Han and Xue have explored the rational design of small hairpin RNAs that access excited states created by strand sliding of one or two base pairs. MD simulations suggested that the single-nucleotide slide within a stretch of RNA helix occurs in a stepwise manner, explaining why the activation free energy of the transition matches the energy cost for breaking only a single Watson–Crick base pair. Moreover, reshuffling kinetics between the ground and excited state are modulated by the stability of the hairpin tetraloop [28]. Recently, developed chemical shift prediction of RNA imino groups based on base-pair triplet motifs was heavily relied on to confirm the conformation of excited states and was as well used for the characterization of an excited state of P5abc ribozyme, including an earlier speculated non-native G•G mismatch [29].

Close insights into RNA–protein interactions

Ribonucleoprotein (RNP) complexes are essential elements of cellular machinery functioning, particularly for the processes of transcription, translation, mRNA processing, and cellular trafficking. The complexity of RNA–protein interactions requires a combination of various analytical methods, including NMR spectroscopy (Figure 1) [2,30–36]. Recently, NMR-derived data on RNA-protein complexes were successfully incorporated into complex integrative approaches to modeling of large RNPs [37].

Classic NMR analysis of RNA–protein interactions includes the detection of chemical shift perturbations (CSPs) upon binding, using assigned heteronuclear spectra of both RNA and protein, which makes it possible to identify interaction surface with residue and even atom specificity. CSP analysis is usually performed on non-crowded spectral regions of heteronuclear spectra, but the information is limited mostly to hydrogen-bonded protons. More information on the interaction surface is provided with NOESY spectra, which are severely crowded. Consequently, different types of heteronuclear edited and/or filtered NOESY spectra using isotopically labeled samples are commonly acquired to remove intramolecular cross-peaks and observe RNA-ligand NOE contacts [31,34,36]. The latter approach enables the extraction of interatomic distances of interacting partners, providing restraints for molecular dynamic calculations of RNP complex model. For instance, using both, CSP and intermolecular NOE contacts, supported by cross-linking experiments and followed by molecular modeling, Allain group unraveled protein-RNA recognition event, involved in spliceosome assembly [36].

A great example of the combined approach using NMR with complementary methods is the study of a complex formed by protein assembly HnRNP A1/A2 with 75K lncRNA. The full-length 75K lncRNA is too large for high-resolution NMR, so the protein-binding fragment was first localized by DMS footprinting of free and bound RNA. A high-resolution structure was calculated using NMR-derived NOE restraints and further refined with residual dipolar coupling data and envelope obtained from SAXS. CSPs upon the titration of RNA with protein revealed multiple loci of HnRNP A1/A2 binding [33].

While the classic approach allows the characterization of structured RNA fragments, the incorporation of ^{19}F-modified nucleotides in RNA enables probing of disordered regions upon the interaction with protein. A combination of molecular modeling and ^{19}F NMR allows insight into the model of large and dynamic RNP [38].

Functional principles of RNA-interacting proteins can be investigated with NMR methods for the detection of dynamics. Hohmann et al. observed refolding of bimodal RNA induced by photolabile group cleavage in the presence of RNA chaperone protein by real-time NMR. Analysis of base-pair stabilities via temperature-dependent solvent exchange rates indicated that chaperone acts by the destabilization of RNA base pairs at specific positions, promoting the formation of a pseudoknot transition state (Figure 3C) [32].

Structural changes of RNA upon interaction with small molecules

Interactions of RNA with small molecules are involved in many biological processes. Indeed, they are tightly

state. (**g**) Modified tRNA^{fMet} displays increased stabilization compared to its non-modified analog. Modifications in the tRNA are designated with the red circles. The figures were adapted from Refs. [1,26,27,32,39,40], and tRNA structure was created with Vienna RNA Websuite.

connected to the adaptive responses of various bacteria, which can occur via conformational change of riboswitch upon binding to low molecular weight ligands. Synthetic riboswitches are being considered for their potential application as regulatory elements of the translation of therapeutic exogenous RNAs. Understanding the interactions of small molecules with various human coding and non-coding RNAs could help to explain many cellular cascades and to control the RNA-related processes with drug ligands [41]. NMR spectroscopy (Figure 1) provides an opportunity for insight into structural transformations of RNA upon interaction [8,42,43]. Comparison of NMR spectra of free and ligand-bound forms of oligonucleotides enables to localize residues involved in the interaction and to estimate the strength of the interaction. The information is often extracted from CSPs of proton and heteronuclear resonances. Such data can be used for the analysis of off−target interactions of ligands developed for the selected biologically relevant RNA secondary structures [44]. The observation of intermolecular NOE patterns, as well as cross-peaks between RNA and the ligand, allows the determination of mutual orientation and conformational changes upon interaction. A sophisticated network of hydrogen bonds and stacking interactions could be formed upon ligand binding, as it was found for a complex of synthetic RNA aptamer with 5-carboxy-tetramethylrhodamine [42] and binding of amilorides to regulatory motifs originating from 5′-UTR of SARS-CoV-2 genome [15].

Interactions with minor conformers within the dynamic ensemble are often critical for the biological functions of RNA. In such a case, a combination of dynamic NMR methods and the classic NMR approach to structural analysis provides the necessary information. For instance, the Al-Hashimi group showed the possibility of ligand binding to the low-abundance excited state of modified HIV-1 TAR oligonucleotide [45]. This approach is especially attractive since it was shown that some excited state conformations are poorly recognized by binding proteins, eliminating the competition for binding between small molecule and protein [46]. Furthermore, the binding of ligand argininamide to HIV-1 TAR RNA was explored to understand how excited states offer different pre-folded conformations with binding constants well below the apparent affinity of the dynamic ensemble [47]. These findings are of great importance for the future of RNA-targeted drug development. While computational screening for potential RNA ligands predominantly relies on the most stable RNA 3D structural models, targeting different members of dynamic RNA ensembles promises to significantly improve the selectivity of ligands and with it control of RNA functions.

Estimation of RNA interactions in intracellular conditions greatly improves the biological relevance of obtained data. Using in-cell NMR, Trantirek and Schwalbe groups showed that 2′-deoxyguanosine-sensing bacterial riboswitch is stable enough in eukaryotic cells to allow for the observation of ligand binding. Moreover, the mode of interaction observed in *in vitro* experiments is preserved in cellular conditions [48].

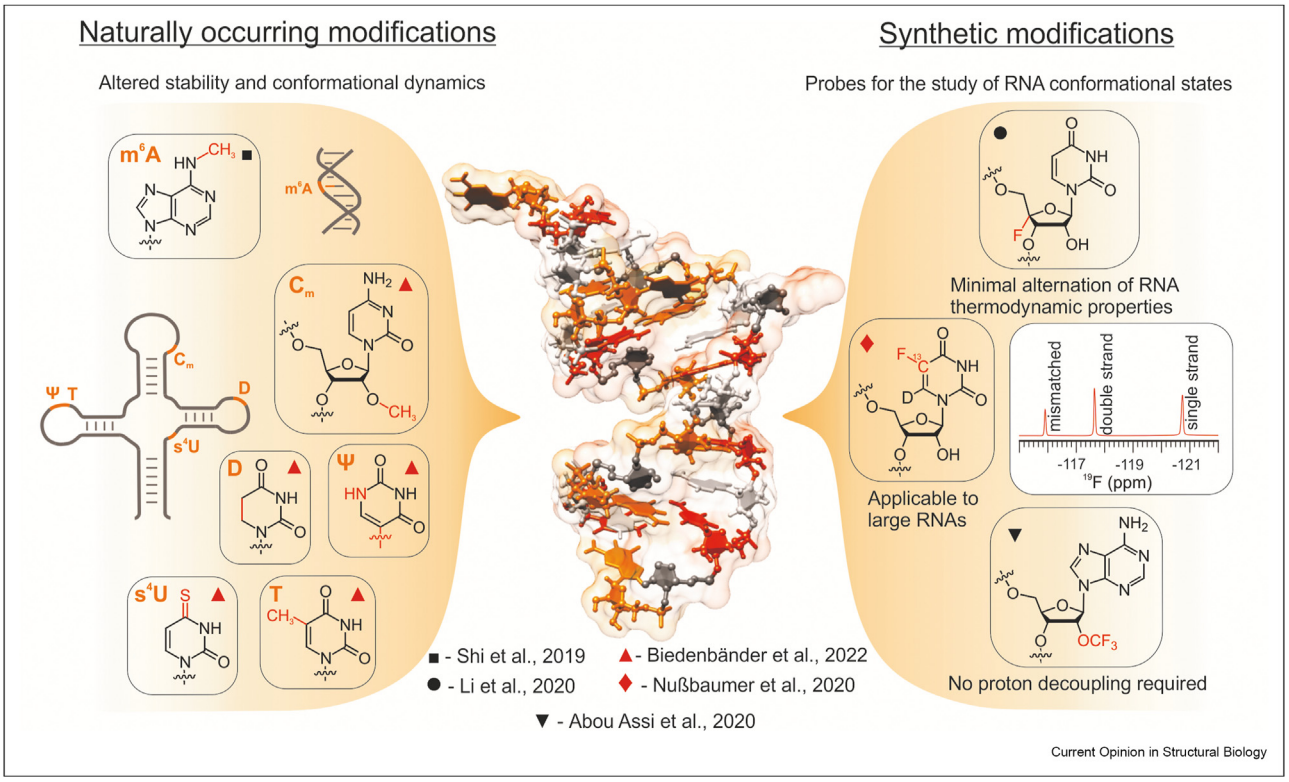
Modified nucleotides: structure probing and altered dynamics

A plethora of modified nucleotides extend the chemical and conformational space for naturally occurring RNAs and influence their interaction capabilities [49]. Modifications are necessary for the proper functioning of cellular machinery, while some appear as a result of RNA damage and are involved in pathological processes. The impact of nucleotide modifications on the structural properties, dynamics, and behavior is currently not well characterized, but with the ever-growing availability of enzymatic and chemical methods for residue and site-specific labeling, it has become an area of lively research, producing fascinating results.

When compared to non-modified analogs, RNAs with modifications demonstrate altered local and global dynamics as well as thermodynamic stability. Therefore, the methods of dynamic NMR are advantageous for their study and are often implemented. For instance, using the chemical exchange saturation transfer (CEST) method, the Al-Hashimi group showed that N6-methyladenosine (m^6A), the most abundant post-transcriptional base modification found in eukaryotic RNA, significantly decreases the rate of annealing, but it does not affect the rate of duplex dissociation [50]. The decrease in annealing rate was further attributed to the exchange of m^6A − U Watson−Crick base pair with a singly hydrogen-bonded low-populated mismatch-like conformation in which the methylamino group is *syn*, and a different preference for *syn*/*anti*-isomer in paired or unpaired form (Figure 3f). Two pathways of isomerization before or after duplex annealing effectively slow down the process and explain why the modification robustly slows diverse cellular processes [40]. Jones et al. further explored m^6A in the context of the A-repeat region of the lncRNA Xist involved in X-chromosome inactivation. Modification in the AUCG tetraloop of Xist was shown to stimulate the binding of a reader protein due to the local unfolding of the corresponding stem (Figure 3d) and shows how modification can affect interaction capabilities with functional outcomes [39].

tRNAs constitute a class of cellular RNAs with the highest modification rates (Figure 4). Modifications are incorporated during the process of tRNA maturation. Observation of the change of imino proton pattern in NMR spectra of transcript incubated with the enzymes revealed that nucleotides of yeast tRNA are modified

Figure 4



Schematic representation of the types and roles of modified nucleotides in NMR studies of RNA. Modified nucleotide symbols: m^6A - N^6-methyl-adenosine, C_m - 2′-O-methylcytidine, D - dihydrouridine, Ψ – pseudouridine, S_4U - 4-thiouridine and T - 5-methyluridine.

not randomly, but in a certain order, and interconnection between particular modification events is present [51]. The detailed structural information obtained by NMR helped to understand how modified nucleotides affect the local and global structure of RNA. Incorporation of the naturally present modified nucleotides in $tRNA^{fMet}$ from *Escherichia coli* was analyzed by NMR methods identifying motion in the pico- and microsecond range (Figure 3g). Base pair opening dynamics were assessed with measurements of solvent exchange rates of hydrogen-bonded protons. Modifications clustered in the core region of $tRNA^{fMet}$ increased fast local movements and lead to the stabilization of the tertiary structure in comparison with the non-modified analog [1].

While naturally occurring modifications are of interest to discover additional layers to RNA function, artificial modifications of nucleotides open perspectives for the study of structure and interactions of RNA that are typically not possible (Figure 4). Fluorine substitutions in nucleobase or ribose moieties are designed in such a manner that the fast estimation of RNA secondary structure using 1D or 2D NMR spectra becomes possible. Instead of complex spectra with highly overlapped resonances, only a few characteristic signals are observed. The information can be extracted from the chemical shifts of fluorine atoms that are responsive to the secondary structure of RNA.

To study biologically relevant RNAs, fluorine substitutions of choice should not significantly affect the structure and stability of RNA. At the same time, obtained ^{19}F spectra should unambiguously report on the RNA state [52–55]. For instance, the combined efforts of the Plavec and Zhou groups resulted in the synthesis and characterization of 4′-fluorinated RNA [54]. 1D ^{19}F spectra revealed the sensitivity of ^{19}F chemical shift on whether modified nucleotide is present in ssRNA, dsRNA, or forms a mismatched base pair.

Remarkable studies were performed using C5-modified pyrimidine residues with a $^{19}F–^{13}C$ spin pair. Kreutz group [55] developed the chemical synthesis of RNA oligonucleotides with $^{19}F–^{13}C$ labeled cytosine and uracil residues and showed their applicability for the probing of RNA secondary structure. Dayie group [53] used T7 *in vitro* transcription to incorporate $^{19}F–^{13}C$ labeled uracil residues and demonstrated their potential in probing not only secondary structure but also

interaction with small molecules. For both groups incorporation of $^{13}C-^{19}F$ spin-pair allowed exploitation of the TROSY effect, circumventing significant line broadening caused by large chemical shift anisotropy of ^{19}F and resulting in well-resolved NMR spectra of RNA constructs beyond 70 nt. The development of ^{19}F-modified oligonucleotides and methods of their incorporation into RNA molecules has a tremendous impact on the progress in the field of in-cell NMR (Figure 1). The simplicity of spectral data and the absence of ^{19}F isotope within cell enable probing of RNA structure and interactions within the crowded intracellular environment [48].

Methodological developments

The pandemic of COVID-19 has seen a rise in interest in RNA structural studies by NMR. Consequently, several new approaches were tested as a result of the efforts to reduce the amount of sample needed and increase speed and sensitivity, such as the use of hyperpolarized water [56] or Hadamard encoding [57,58]. In line with speeding up data acquisition, Nichols et al. have investigated the fidelity of eNOEs measured at decreasing non-uniform sampling densities and created guidelines for an optimal NUS density based on the number of cross-peaks needed for reconstruction [59]. In the field of dynamic NMR methods, ^{15}N CEST NMR experiments were adopted to utilize protonated and non-protonated nitrogens as representatives of hydrogen-bond donors and acceptors, complementing ^{13}C CEST profiles in detecting the excited conformational state of the fluoride riboswitch [60]. ^{13}C CEST experiments were further pushed to detect faster motions using high-power radio-frequency fields [61]. These studies were complemented with a detailed assessment of the effect of carbon–carbon coupling on relaxation parameters measured in samples with different labeling schemes [62] and with quantification of the effect of ^{13}C dipolar coupling on relaxation rates, which become non-negligible, especially at the higher magnetic field and large molecular weights of RNAs [63]. The high-pressure NMR was also shown to be useful for the detection of low-abundance excited states of RNA [64].

The need and desire of the NMR community to obtain structures and structural ensembles faster fuels lively interest in improved force–field parameters [65,66] for MD simulations of nucleic acids and procedures for more efficient implementation of NMR data. Several approaches with a combination of structure prediction programs, MD and NOEs, or chemical shift information were used to produce structural ensembles of HIV-1 TAR [67] or SINEB2 RNAs [68]. Chemical shifts were suggested to be used to refine RNA structures [69] or decode structural features, such as solvent exposure, base stacking, or base pairing [70]. Liu et al. have identified, designed, and assigned sequences with no or limited chemical shift information to help populate the Biological Magnetic Resonance Data Bank [71]. For a more thorough description of the integration of solution-state NMR with computational techniques that aim at the improved and faster description of dynamic RNA ensembles, the reader is directed to the recent review by Liu et al. [72].

Solid-state NMR

Solid-state NMR experiments yield atomic resolution structural information without molecular weight limitations and are, therefore, attractive for large RNAs and RNA-protein complexes. Aguion et al. described a set of experiments for the identification of complete nucleobase spin systems on uniformly labeled RNA samples, promising to provide rapid access to RNA secondary structure by ssNMR at high spinning speed in protein–RNA complexes of any size [73]. Ahmed et al. proposed an ssNMR approach that yields the structure of protein–RNA complexes by measuring paramagnetic relaxation enhancement effects evoked on the RNA with a paramagnetic tag coupled to the protein [74].

Smart isotopic labeling is relevant also for ssNMR. For example, differential isotope-labeled ribozyme and substrate strands in combination with magic-angle spinning (MAS) dynamic nuclear polarization NMR were used to investigate structurally and functionally relevant inter-strand and inter-stem contacts [75]. MAS NMR has been used to investigate ^{14}N spectra of G-quartets, using $^{1}H \rightarrow {}^{14}N \rightarrow {}^{1}H$ double cross-polarization to obtain 2D spectra and identify inter-plane dipolar contacts between the stacked G-quartets [76].

Conclusions

The development of therapeutic approaches involving RNA requires a deep understanding of its behavior in a cellular environment. Since the majority of RNA in living cells are large polynucleotide chains, a significant shift of focus toward long RNA fragments is observed, which is imminently connected to the increasing complexity of spectral data. Biological relevance also requires the understanding of the conformational ensemble of RNA fragments rather than of a single most populated state only, as well as of its interconversion upon interactions with proteins and ligands. The study of large and dynamic complexes forming in the cellular environment as a result of such interactions brings new challenges for the NMR field and requires its integration with various complementary approaches. On the other side, in order to maximize the biological relevance of obtained data, methods developed for *in vitro* models are being adapted for the study of *in-cell*-mimicking or intracellular conditions. Moreover, changes in the dynamics and structural aspects of modified RNAs are currently intensively explored. Possible future studies

will focus on the intriguing topic of how the local dynamics interfere modulate and direct the incorporation of the modified nucleotides themselves. All these trends cause intensive efforts to improve NMR resolution and simplify the procedures for probing structure and interactions, as well as develop methods for the study of the dynamic behavior of RNA.

Glossary box

Chemical exchange saturation transfer (CEST) is one of the NMR experiments used to detect dynamic events through measurements of spin relaxation rate at different magnetic field strengths. The typical timescale detected is in the μs range with populations of excited states as low as 1%.

Chemical shift perturbation (CSP) is a technique that uses a difference in chemical shifts upon binding to demonstrate complex formation, locate the binding site, and measure binding affinity.

Dimethyl sulfate (DMS) footprinting is a technique used to study the structure and interactions of nucleic acids. DMS is used to methylate non-base-paired residues, after which methylation sites are detected via mutations in a reverse transcription reaction.

Dynamic nuclear polarization (DNP) is a method that transfers large spin polarization from electrons to nearby nuclei using microwave irradiation, effectively increasing the sensitivity of NMR spectroscopy.

N**uclear Overhouser Enhancement (NOE)** is one of the most informative and widely used NMR observables for NMR structure determination. It is based on the dipol−dipol interaction between two nuclei that are close in space and, therefore, provides information on the distance between nuclei.

Exact nuclear Overhauser enhancements (eNOE) take into account certain limitations that cause conventional NOE to be only semi-quantitative. Thus, eNOE allows to extract exact distances and futhermore, to convey not only structural but also dynamic information.

Excited state is a low abundance (as low as 0.01%) and short lifetime (μs) conformational state within the dynamic ensemble of nucleic acids.

Magic-angle spinning (MAS) exploits sample spinning at high frequency at a magic angle with respect to the magnetic field to produce better resolution ssNMR spectra with linewidths similar to solution-state NMR.

Molecular dynamics (MD) simulations provide information on the vibration and other physical movements of atoms and molecules using ball-and-stick model defined by force field parameters.

Non-uniform sampling (NUS) is an acquisition method for 2D and higher dimensional NMR experiments that only samples a subset of the indirectly detected points resulting in a substantially reduced measurement time.

Paramagnetic relaxation enhancement (PRE) is a technique that provides distances based on the increased relaxation rates of nuclei that are close to the spin label—a molecular entity with an unpaired electron. Typical distances are in the range of 15−24 Å.

Real-time NMR designates a collection of NMR experiments that detect slow dynamic events on a timescale of seconds and longer, for example, molecular refolding or enzymatic reactions. Typically, the reaction is triggered by the change of environment or chemical cleavage of inactivated (stabilized) part of the molecule.

Relaxation dispersion is a collection of NMR methods used for the identification and characterization of excited states with exchange rates from μs to s. Their main advantage is that they offer information about the structure of the excited states with atom resolution.

Selective 2′-hydroxy acylation and primer extension (SHAPE) is an RNA structure probing technique that chemically modifies RNA as a function of 2′-OH group reactivity. The extent of chemical modification is detected by the primer extension essay.

Small-angle neutron scattering (SANS) and small-angle X-ray scattering (SAXS) are techniques that use neutron and X-ray scattering at small scattering angles, respectively. They report on the size and shape of macromolecules and their size distributions. Used in combination with NMR, they can help orient small fragments within the large RNA.

Solid-state NMR spectroscopy (ssNMR) is a technique for characterizing atomic-level structure in solid materials, such as powders, single crystals, and amorphous samples and tissues.

Conflict of interest statement

Nothing declared.

Data availability

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

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