NMR Tools to Decipher Dynamic Structure of RNA

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Abstract It is now well established that RNAs exhibit fundamental roles in regulating cellular processes. Many of these RNAs do not exist in a single conformation. Rather, they undergo dynamic transitions among many different conformations to mediate critical interactions with other biomolecules such as proteins, RNAs, DNAs, or small molecules. Here, we briefly review NMR techniques that describe the dynamic behavior of RNA by determining structural, kinetic, and thermodynamic properties.

Keywords NMR Dynamics, RNA, Ensemble, Relaxation Dispersion, Base-Pair Kinetics

Dynamic Nature of RNA

Non-coding RNAs[1,2] exhibit a wide range of biological roles including transcription,[3,4] translation[3] and gene silencing.[5,6] What is intriguing is that the 3D structure of RNA, which is simply composed of four nucleotides with similar physiochemical properties, cannot completely explain the diverse functions of the non-coding RNA. Increasing number of studies present evidence that RNA constantly undergoes conformational changes induced in response to cellular cues such as pH, temperature, metabolites, proteins, and RNAs.[7-15] These changes involve re-arrangements in secondary structure that expose functional motifs;[16,17] tertiary contacts that stabilize 3D structure that exerts a function;[18,19] and opening/closing of unpaired residues.[13,20] This conformational flexibility provides the insights into the functional complexity of RNA.

A well-known example of dynamic regulatory RNAs is riboswitches.[10,16] Riboswitches are composed of an aptamer domain where a metabolite binds and an expression platform which regulates transcription or translation. Binding of the metabolite to the aptamer domain ultimately induces the changes in the secondary structure of the expression platform that facilitates or inhibits the transcription or the translation, thus controlling gene expression through modulation of RNA dynamics (Fig. 1A).

The interaction between RNA and proteins to form RNA-protein complexes (RNP) is also a dynamic process that often induces sequential changes in RNA structure upon binding of proteins. These conformational changes of RNA induced by proteins can direct the order of assembly of RNP. This event is prominent in the hierarchical assembly of a ribosome, a ribozyme composed of RNA and proteins.[21] For example, the binding of ribosomal protein S15 to 16S ribosomal RNA re-orients the helices of 16S RNA that favors the subsequent binding of ribosomal protein S6 and S18.[22]

A global conformational change in the secondary structure of RNA is not always a pre-requisite for the function of RNA. A flipping of local bases can regulate the biological processes. For example, 16S ribosomal A-site RNA selects an appropriate

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codon-anticodon helix formed between aminoacyl tRNA and messenger RNA (mRNA) by two adenine bases in its internal loop (Fig. 1B).[23-25]
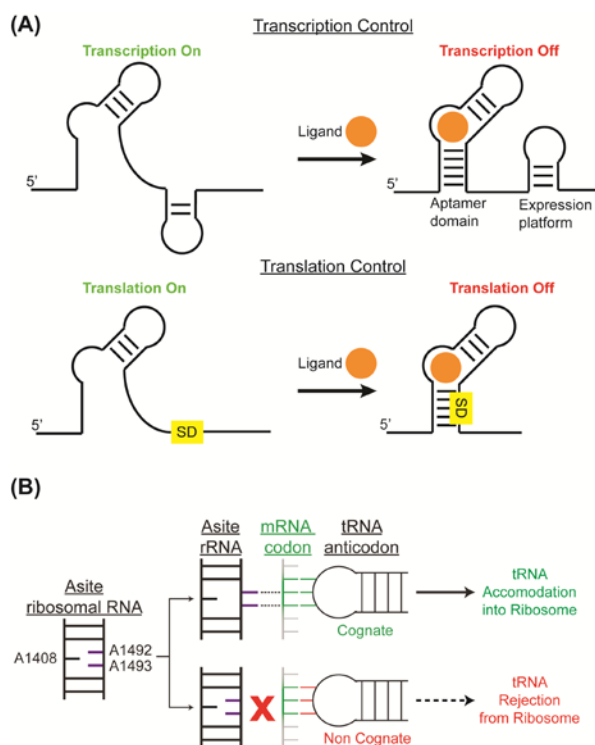

Figure 1. RNA dynamics in biology. (A) Structural change of a riboswitch upon binding of a ligand controls transcription or translation.[16] (B) Recognition of cognate tRNA by a ribosome is determined by two adenine bases in the A-site ribosomal RNA.

There are growing list of regulatory RNA elements involved in pathogenesis including cancer, genetic disorders, viral and bacterial infections, and neurodegenerative diseases.[26] RNA viruses have regions of non-coding RNAs in the 5'- or 3'-ends of their genomes, which regulates the viral life cycle. Many riboswitches regulate bacterial life cycle. MicroRNAs are deficient or over-expressed in carcinoma, acting as biomarkers to diagnose a tumor.[27] Thus, characterizing the structural dynamics of these non-coding RNAs offers novel targets to develop therapeutics.

NMR Tools to Characterize RNA Dynamics

NMR is a powerful tool to characterize the structure[28] and dynamics over timescales ranging between picoseconds (ps) to seconds (s) (Fig. 2). NMR parameters such as spin relaxation, residual dipolar couplings, or chemical shifts are used to characterize the motions by employing different NMR experiments. For example, ^{13}C or ^{15}N relaxation experiments can probe the fast motions with timescale ranging from picoseconds to nanoseconds. Relaxation dispersion experiments can probe much slower motions (microseconds to milliseconds). Interactions among biomolecules such as ligand binding and structural re-folding are typically occurring at the timescale of microseconds to seconds.[29-33] Thus, the motions occurring at this timescale are of great interest to understand the function of the regulatory RNA.
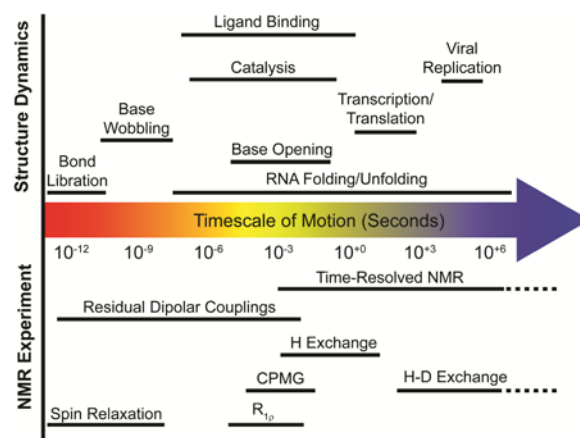

Figure 2. NMR tools to probe structure dynamics of RNA. Timescale of motion are shown from picoseconds (10^{-12}) to a million seconds (10^{+6}). The structure dynamics of RNA in biology and the NMR experiments to probe them are shown.[34]

Unlike proteins, RNA has strong carbon-carbon scalar couplings, narrow window of chemical shifts, and low number of protons. These structural discrepancies often challenge the implementation of NMR experiments, which are mostly optimized for proteins, and the interpretation of NMR parameters. Here, we will briefly review the NMR experiments to probe the μs-s dynamics of RNA.

Relaxation Dispersion

The relaxation dispersion provides in detail the conformational exchange occurring from microseconds to milliseconds.[35-37] This experiment modulates the contribution of exchange broadening to the transverse relaxation rates and can deduce the exchange rate constants (k_{ex}) among exchanging conformations, population (~1-50%) of each conformation, and the chemical shifts that describe the structure of each conformation. There are two major limitations on this method. First, the exchanging conformations must have distinct differences in chemical shifts. If two conformations have similar chemical shifts, the relaxation dispersion cannot detect the conformational exchange between them. Second, an exchanging system with three or more states is often challenging to interpret the relaxation data. Few studies including numerical fitting to the Bloch-McConnell equations are aimed to accurately describe the multi-state kinetic models.[37-39]

Two experiments, Carr-Purcell-Meiboom-Gill (CPMG)[40,41] and R1ρ,[42-44] are frequently employed in the relaxation dispersion study. By modulating repeating frequency of a refocusing pulse in CPMG (Fig. 3A) or spinlock powers in R1ρ (Fig. 3B), we can control the peak broadening due to chemical exchange on the observed peak of interest. When there is a chemical shift difference due to conformational exchange, the transverse relaxation rate will differ by modulating powers (Fig. 3C). To obtain kinetic and thermodynamic information on the conformational exchange, we perform CPMG experiments and complementary NMR experiments (HSQC/HMQC) in two different magnetic fields,[45] or R1ρ in various offsets and spinlock powers. The exchange rate constants, chemical shift differences between the ground state and the transient state, and population of the transient states can be obtained in a single magnetic field by globally fitting the R1ρ data, collected in various offsets, to a relevant kinetic model.[37] The ^{13}C or ^{15}N chemical shifts of RNA are great indicator of the conformation[42] including hydrogen bonding,[46] syn- or anti- base conformation,[47] base stacking,[48] sugar pucker,[49] and protonation.[50] Thus, we can depict the secondary structure of the transient states by observing the changes in ^{13}C and ^{15}N chemical shifts derived from the relaxation dispersion.
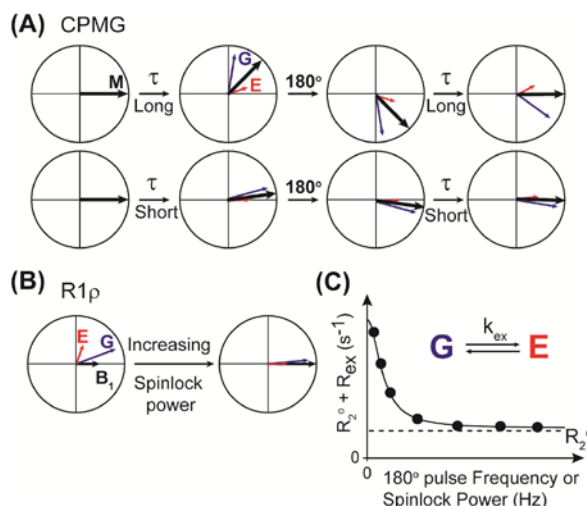

Figure 3. Principles of relaxation dispersion. Underlying principles of CPMG (A) and R1ρ (B) relaxation dispersion are briefly illustrated. (A) the chemical exchange between ground state (denoted as G) and transient state (denoted as E) is controlled by a frequency of the applied refocusing pulse (180°). (B) The chemical exchange is controlled by modulating spinlock field (B_1). (C) When there is a chemical exchange, the effective transverse relaxation rate ($R_2^o + R_{ex}$) decreases by a function of refocusing pulse frequency or spinlock power. Increasing the frequency of a refocusing pulse or spinlock power converges the effective transverse relaxation to the intrinsic transverse relaxation rate (R_2^o).

For RNA, R1ρ relaxation dispersion experiment is preferred over CPMG. CPMG cannot suppress the strong carbon-carbon coupling in nucleotides that interferes with the chemical exchange.[51] It is almost impossible to selectively refocus a carbon nucleus in RNA with a hard pulse, unless the RNA is selectively isotope-labeled.[52,53]

Characterizing Dynamic Ensemble of RNA Using Residual Dipolar Couplings

Many regulatory RNA elements contain

noncanonical base pairs or unpaired bases, which form a bulge or an internal loop. These structures twist and bend the helical structure to accommodate ligand binding. The motion of interhelical bend and twist cannot be fully characterized by spin relaxation, because the motional timescale of interhelical bend/twist is much slower than the overall tumbling. To quantify the interhelical motions, residual dipolar coupling (RDC) is used.

RDC is a parameter that depends on the angle between a chemical bond and the external magnetic field.[54,55] The angular dependency of RDC can be described using the following term, $< (3 \cos^2 \theta - 1) / 2 >$, where the angular bracket represents the time-average of chemical bond orientations observed at a rate faster than the dipolar coupling, and the θ is the angle between the chemical bond vector and the external magnetic field (Fig. 4). Under isotropic condition, the angular term averages out to zero, making RDC invisible on the spectra. However, by introducing a small degree of alignment on the RNA molecule, the angular term is no longer averaged to zero, and the resonance on the resultant spectra becomes a doublet split by the amount of RDC. The RDC values are experimentally calculated by subtracting the splittings in the presence and the absence of the alignment media (Fig. 4).

The partial alignment is experimentally introduced by dissolving RNA molecule in the alignment media. A typical media used for RNA is a filamentous bacteriophage, pf1[56] (Fig. 4). The bacteriophage that has a rod-like shape, is negatively charged to avoid unwanted interaction with the RNA, and is tolerant to the high ionic strength in solution. The ideal degree of alignment is 10^{-3}, meaning one in every 1,000 molecules are completely aligned. If the degree of alignment is less than 10^{-5}, the resulting RDC is too small to be analyzed on the spectra. If the alignment is larger than 10^{-2}, the RDC values become unfavorably large to analyze large RNA with sufficient spectral resolution. Typically, 15-25 mg/ml of pf1 is used to align the RNA molecules, and the concentration of pf1 in the NMR sample can be quantified by dividing the ^2H residual quadrupolar splitting by a constant factor of 0.886.[56] Ideally, five

independent sets of RDC values in different alignment media are desired[57] to characterize interhelical motions in greater spatial resolution. The alignment of proteins can be changed by using different alignment media,[58,59] but the alignment of RNA hardly changes under different alignment media.[60] This is due to the polyanionic and cylindrical nature of RNA which simplify the interaction with the alignment media.[61] The modulation of the RNA alignment can be achieved by elongating the RNA helices[62] or introducing a bulge to kink the helical structure.[63]
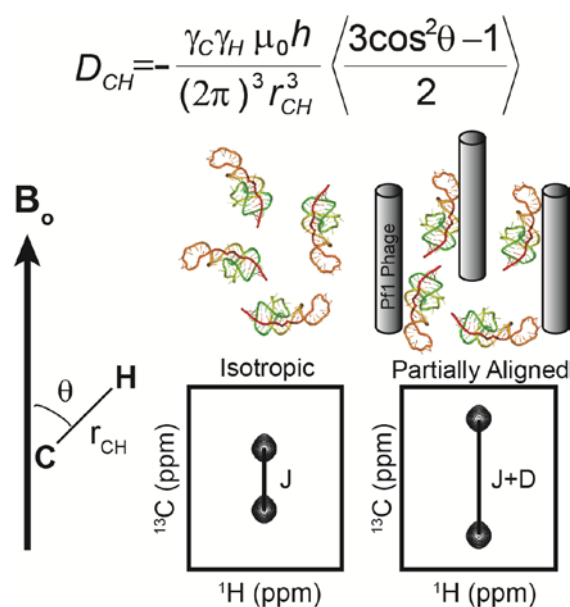

Figure 4. Measurement of residual dipolar couplings. Definition of dipolar coupling of C-H chemical bond. θ denotes the angle between the chemical bond and the external magnetic field (B_o), r denotes the bond length, γ is the gyromagnetic ratio of a nuclei, μ_o is the permeability in a vacuum, and h is planck's constant. The angular bracket represents time- and ensemble-average. The residual dipolar coupling (D) is measured by subtracting the splitting in isotropic condition from the splitting in partially aligned condition.

The time-averaged RDCs contain contributions from internal motions and overall motions. Probing the internal motion without considering the overall motion is often difficult in RNA, which is highly flexible. Decoupling of these motions can be achieved by elongating the RNA helix.[62] The

elongated RNA helix can define the overall alignment of RNA and simplify the interpretation of RDCs to probe the internal motions in RNA.

RDC can sense a broad timescale of motion from picoseconds to 10 milliseconds. The molecular dynamics (MD) simulations, which can provide atomic-level description of RNA motions up to low µs, can synergize with RDCs to provide the ensemble-based description of RNA dynamics.[64,65] A set of RNA conformers that recapitulates the experimental RDC values is selected from a pool generated by MD simulation. The dynamic ensemble of transactivation response element (TAR) from HIV-1 was constructed in this manner and contained many conformations that closely resembled the ligand-bound states, supporting the mechanism of ligand recognition by conformational selection.[66] Virtual screening targeting the dynamic ensemble of TAR yielded a small molecule that inhibited the replication of HIV-1 in cells with IC_{50} of ~20 µM.[67]

Base Pair Dynamics

Many regulatory RNAs undergo dynamic re-arrangement of secondary structure, forming or losing base pairs. For decades, proton exchange experiment has been a simple but elegant method which can probe the base pair dynamics with a site-specific resolution.[68] Proton exchange experiment exploits the fact that when base pairs are open, imino or amino group of bases can exchange its protons with water[69] (Fig. 5) and this exchange perturbs the NMR signal. The slow exchange dynamics in a timescale from few seconds to hours can be probed by proton-deuterium (H-D) exchange experiment.[70-72] The RNA in H_2O buffer is rapidly exchanged into a D_2O solvent and the disappearance of imino or amino protons are monitored by 1D or 2D spectra in a time-dependent manner. The fast exchange dynamics with a timescale of motion from millisecond to second can be probed by selectively perturbing the water resonance and monitoring the magnetization transfer to exchangeable protons in RNA during a mixing time.[73] A base catalyst such as

Tris and NH_3 is titrated to the sample. Kinetic parameters including opening/closing rate constants and equilibrium constant can be extracted by analyzing the data in the function of the base catalyst. These parameters provide a detailed description of base pair opening kinetics.
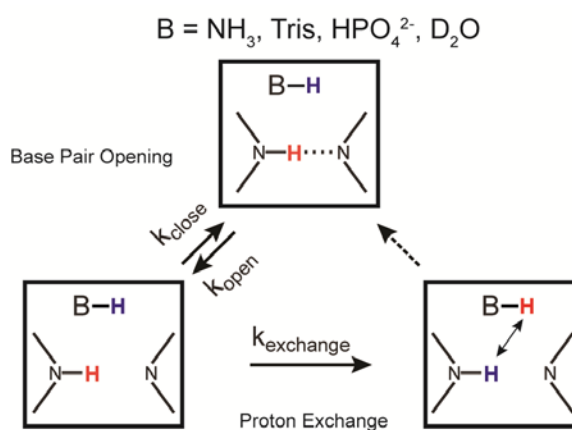

Figure 5. Kinetic scheme of proton exchange experiment. The proton exchange is a two-step process. First, the base pair is in equilibrium of open state (k_{open}) and closed state (k_{close}), and the proton in the base catalyst (denoted as B) is exchanged ($k_{exchange}$) with the exposed proton in the base pair.

Proton exchange experiments are best suited for studying the secondary structure or the tertiary structure of RNA. The experiments have been implemented to study the base-pairing network in transfer RNA,[70-72,74] ribozyme[75] and RNA-protein complex.[76,77] The proton exchange experiments can complement the relaxation dispersion by probing flexible base pairs which may be a site for secondary structure transition.

Perspectives

Dynamics studies by solution-state NMR have revealed complex and dynamic structure landscape of regulatory RNA. New NMR methodologies are being developed to address two major challenges in the current dynamics studies on RNA. First, current experiments cannot thoroughly describe the

conformational dynamics of RNA with an atomic resolution. To determine the transient structures with a lifetime of few micro-to-milliseconds, a method to measure RDCs of the transient states has been developed.[78] Paramagnetic relaxation enhancement[79] and the solid-state NMR[80,81] can be also utilized to characterize the transient structures of RNA. All the NMR experiments including the ones described in this review, and additional NMR parameters can complement each other to improve the temporal and spatial resolution of RNA behavior. The other challenge in RNA dynamics is to correlate the dynamic behavior of RNA with its function. Currently, the link between the RNA dynamics and the function is qualitative, even though the RNA sequence and secondary structure often show evolutionarily conserved dynamics. Therefore, we need to systematically and quantitatively investigate how RNA sequence and cellular cues influence the dynamic landscape of RNA by determining dynamic structures along with thermodynamic and kinetic parameters. This will provide unprecedented discoveries and profound insights into the fundamental behavior of RNA.

Acknowledgements

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