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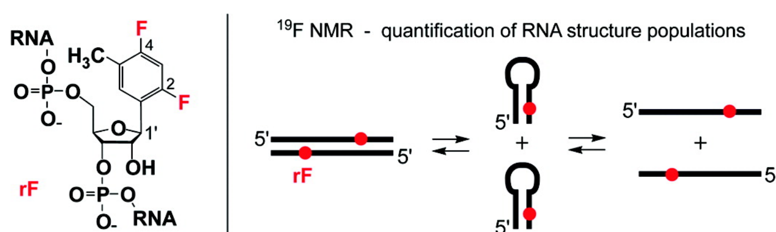

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^{19}F NMR Spectroscopy for the Analysis of RNA Secondary Structure Populations

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RNA offers a functional repertoire that often relies on its intrinsic conformational flexibility to adopt alternative secondary structure elements. Several recent examples for RNA dependent regulation mechanisms can be rationalized by the interplay between the accessibility of a single-stranded region in one RNA fold, whereas sequestration in a double helix occurs in the alternative.[1,2] In a molecular perspective, the conformational interplay between single strand and double helix is reflected by the different conformations that an oligonucleotide of self-complementary (or partly self-complementary) sequence can adopt. These conformations are intermolecular duplexes and intramolecular stem−loop structures (hairpins) beside unpaired and therefore largely unstructured single strands.

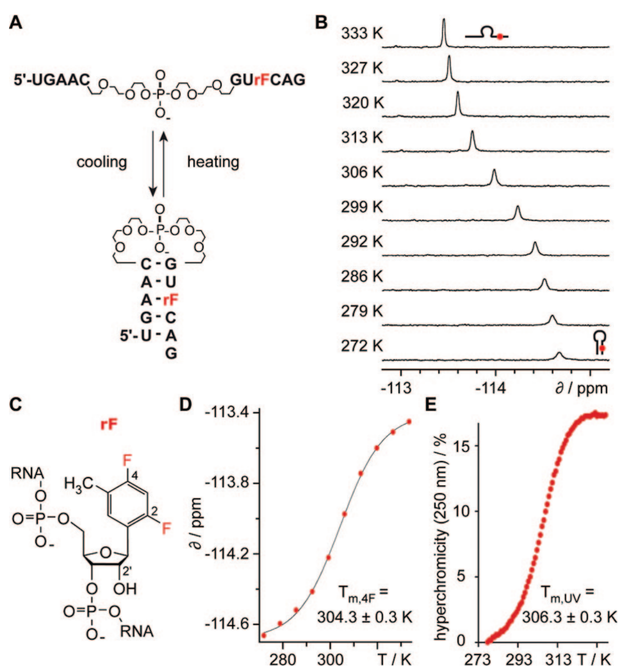

Figure 1. Monomolecular melting transition of a fluorine-labeled RNA: (A) RNA sequence; (B) ^{19}F NMR spectra at different temperatures; (C) fluorine-modified nucleobase as uridine replacement; (D) melting profile derived from chemical shift dependence ∂_{4F} on increasing temperature (c_{RNA} = 0.3 mM; 25 mM Na_2HAsO_4, no additional salt, pH 6.5); (E) UV melting profile (c_{RNA} = 8 μM; 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.0).

Even for short oligonucleotides, the experimental verification, in particular, quantification of the individual conformational populations can become a complex task.[3] Commonly, native gel electrophoresis and UV melting profile analysis[4] are applied to distinguish between mono- and bimolecular structures. Here, we demonstrate that one-dimensional ^{19}F NMR spectroscopy of oligonucleotides with fluorine labels is a useful and straightforward approach to obtain direct information on coexisting nucleic acid structures.

The original concept was to analyze oligonucleotides of defined conformational behavior first, on the one hand hairpin systems that show no competing duplex formation and, on the other hand, duplexes that are composed of strands whose sequences have no propensity for hairpin formation. In a second stage, if correlation of ^{19}F NMR resonances to temperature dependent conformational changes were successful, oligonucleotides with the inherent propensity to adopt mixtures of secondary structures would be analyzed.

An important question of experimental design was the kind of fluoro labels to be used. Out of a variety of possible sites for NMR appropriate fluoro labeling of RNA (e.g., ribose 2′-F,[5] 5-F pyrimidine,[6] 2-F adenosine,[7] 5′-CF_3 pyrimidine,[8a] 5′-alkyne-C$(CF_3)_3$ pyrimidine[8b]) we decided in favor of the uracil mimetic 2,4-difluorotoluene (nucleoside rF).[9] The corresponding U to rF replacements have been recently applied in siRNA approaches and provided additional motivation to develop a tool with potential application in the conformational analysis of rF modified siRNAs.[10]

Figure 1 illustrates the analysis of a short RNA double helix that is linked by ethylene glycol units on one helix end (Figure 1A). Such RNAs are known for an ideal monomolecular melting behavior.[3e] Upon stepwise increase of temperature, the ^{19}F NMR spectra reflect this behavior by a pronounced shift of the 4-F (Figure 1B,C) and 2-F resonances (Supporting Information) demonstrating that the melting process occurs in the fast exchange regime on the NMR time scale. Plotting chemical shift values against temperature results in a sigmoid curve (Figure 1D). Importantly, the corresponding derivate curve (or alternatively, by applying a sigmoid fit function) provides the T_m value with good precision for RNA at concentrations that are generally too high to be accessible for UV melting analysis. In the present case, the NMR derived T_m value (low salt concentration) was 2 K lower compared to the UV derived T_m value (high salt concentration) (Figure 1E). As expected, the ^{19}F NMR-based determination of T_m values is sensitive to varying salt conditions, and T_m values obtained by the two different methods under similar salt conditions compare well for the monomolecular, concentration-independent transition of this RNA hairpin (Supporting Information). The melting behavior of a bimolecular RNA double helix analyzed by ^{19}F NMR spectroscopy is illustrated in Figure 2. The sequence of the duplex has been designed for an ideal two-state transition (Figure 2A). In contrast to the monomolecular melting process discussed above, the observed spectra show that melting of the bimolecular duplex is in the slow exchange regime on NMR time scale (Figure 2B). Consequently, at temperatures close to the melting temperature, two sets of signals are observed: one for the rF label within the duplex and one for the rF label within the free single strand. The T_m value can be estimated by plotting the fraction of single strand against temperature and then applying a sigmoid curve fit (Figure 2D). The T_m values obtained by UV melting for this bimolecular melting process

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compare well when extrapolated to RNA concentrations typical of NMR studies (Supporting Information).

Having demonstrated the ^{19}F NMR behavior of hairpins and duplexes, we proceeded with self-complementary sequences that are prone to exist in a mixture of structures. In the case of 5′-CGCrFAAUUGGCG we observed a pronounced shift with sigmoid character for the 2-F ^{19}F resonance indicating hairpin formation throughout the whole temperature region measured (Figure 3, left). In the case of 5′-CGCrFAAUUAGCG we observed a distinctly different signal pattern (Figure 3, right): the constitutional change of rF:G to rF:A resulted in a second set of 2-F and 4-F resonances at low temperatures indicating significant duplex over hairpin competition. The comparison of these two oligonucleotides nicely exemplifies the convenience of the ^{19}F NMR analysis presented here. Although basic characterization of such structural equilibria can be performed by gel shift assays or UV melting profile analysis the advantage of the ^{19}F NMR approach is direct quantification of duplex/hairpin populations in a concentration range that is hardly accessible by the alternative methods.

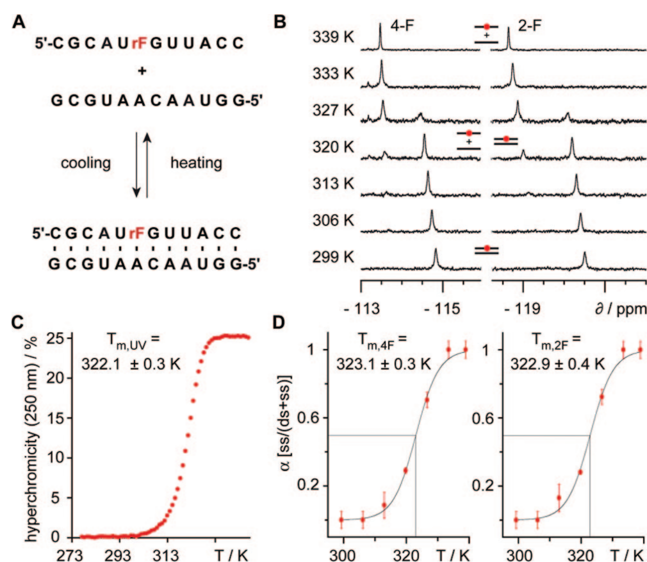

Figure 2. Bimolecular melting transition of a fluorine-labeled RNA: (A) RNA sequence; (B) ^{19}F NMR spectra at different temperatures; (C) UV melting profile ($c_{RNA} = 16\ \mu$M; 10 mM Na_2HPO_4, 150 mM NaCl, pH 7.0); (D) melting profile derived from α/T graph of ∂_{4F} and ∂_{2F} ($c_{RNA} = 0.6$ mM; 25 mM Na_2HAsO_4, no additional salt, pH 6.5).

From a NMR spectroscopic point of view, the approach is user-friendly compared to the analysis of structure equilibria of non-labeled RNA by ^{1}H NMR spectroscopy that suffers from severe signal overlap. Moreover, when detection occurs via imino protons of Watson Crick base pairs the reliance on exchangeable NH−N nuclei is disadvantegeous for the quantification of structure equilibria, for investigations at elevated temperatures, or for investigations at different buffer conditions that affect H/D exchange rates. Compared to other ^{19}F NMR studies of nucleic acids, the present application is a first example how temperature dependent shifts of ^{19}F resonances can be advantageously used for the analysis of dynamic RNA secondary structure equilibria.

^{19}F labeling of RNA with single nonexchangeable fluorine atoms has become straightforward in recent years and has become an integrated part of engineered functional RNA with therapeutic potential, for example, for aptamer, ribozyme, and siRNA technologies. We have furthermore demonstrated the broad applicability of the approach for DNA structure equilibria and exemplarily for another type of fluoro label. The latter is of relevance for specific

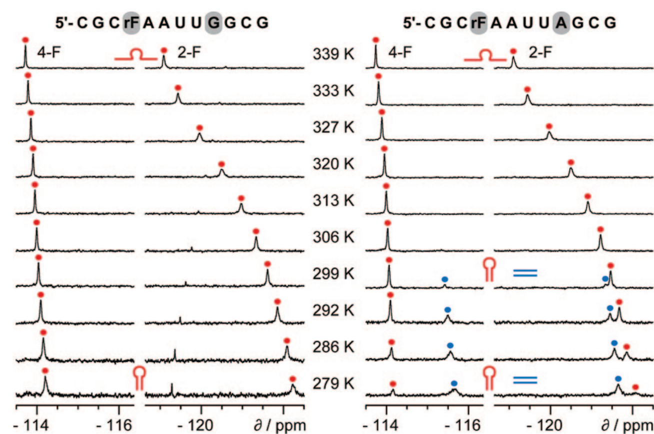

Figure 3. Structure equilibria of self-complementary RNAs. ^{19}F NMR spectra at different temperatures; $c_{RNA} = 0.3$ mM; 25 mM Na_2HAsO_4, no additional salt, pH 6.5; for interpretation see main text.

applications that are incompatible with the decrease in thermodynamic stability resulting from an A:U to A:rF replacement (Supporting Information).

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Supporting Information Available: Synthesis of rF; analysis of other rF/dF/5FU RNAs/DNAs; UV melting analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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