RNA Structures

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Investigation of RNA–Ligand Interactions by ^{19}F NMR Spectroscopy Using Fluorinated Probes**

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Ribonucleic acid (RNA) is now recognized as playing a key role in many biological functions, and is emerging as an important new drug target.[1] However, its therapeutic potential is still underexploited.[2] Indeed, the limited understanding of the interactions between small molecules and RNA still hampers rational drug development of RNA-targeting molecules. Among the different methods available to investigate binding between small molecules and RNA,[3] NMR spectroscopy is particularly attractive as it can deliver information on molecular interactions at the atomic level, including conformational rearrangements that can occur before or upon binding.[4] The dynamic nature of this interaction is particularly important in RNA-regulated pathways.[5]

Many NMR spectroscopy techniques have been developed to visualize dynamic RNA–ligand interactions, most of them based on the observation of either the target or ligand ^{1}H nuclei.[6] However, some difficulties can occur when studying larger strands of RNA, as the number of detectable signals will increase. Introducing a specific label is one way to overcome this problem. An elegant method based on ^{19}F NMR spectroscopy was proposed some years ago by Micura and co-workers. Introduction of a fluorine atom at a specific position of RNA allows local monitoring of binding events at this site.[7] One technical difficulty with this approach is the need to chemically modify the RNA, which can be difficult for large RNAs or for RNAs with modified nucleotides. Furthermore, this modification can affect RNA–ligand interactions.

We have recently shown that ^{19}F NMR spectroscopy can be used to monitor the binding of racemic fluorinated molecules to various RNAs and that chiral recognition can be used to monitor the local conformation of the binding site.[8] The use of ligand-based binding-competition NMR screening using fluorinated ligands has been described by Dalvit for the investigation of protein–ligand interactions (FAXS technique),[9] but has not been applied to the study small molecules interacting with RNA. We report herein that competitive binding of fluorinated probes can be used to detect and quantify the interaction between unlabeled RNA and non-fluorinated ligands and to monitor dynamic RNA folding events (Figure 1).

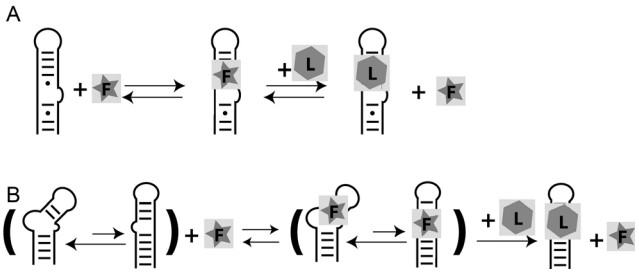

Figure 1. Principle of the displacement experiments. A) Competition for binding to a structured RNA between a fluorinated probe (F, star) and an RNA ligand (L, hexagon). B) Competition for binding to a bistable RNA. In both cases, the fluorinated reporter is monitored by ^{19}F NMR.

The binding of aminoglycosides with 16S23 RNA, a 23 nucleotide hairpin that mimics the decoding A-site of 16S ribosomal RNA, was first investigated as a case study. Although absolute K_D values are difficult to compare since experimental conditions significantly vary from one study to another, the K_D values of the most studied ligands of 16S A-site RNA can be ordered as follows: neomycin < paromomycin < neamine < paromamine, neomycin being the strongest binder and paromamine the weakest.[10] Fluorinated compound **1** being an analogue of desoxystreptamine (DOS), the core moiety of most aminoglycosides, we first checked that it bound to the 16S23 RNA.[11] Its binding was monitored using both ^{19}F and ^{1}H NMR spectroscopy (Figure 2). A K_D of 2 mM was determined by NMR titration. As reported for DOS,[12] compound **1** interacts with two equivalent binding sites of 16S23 RNA in a fast-exchange regime.[13]

We then conducted ligand-based binding competition using compound **1** as a fluorinated spy probe and known binders of 16S23 RNA (neamine **2**, paromamine **3**, neomycin **4**) as competitors. Progressive displacement of the fluorinated reporter could be observed by increasing the concentration of the competitor (Figure 3), the K_D value of paromamine, neamine, and neomycin for 16S23 could be estimated in the

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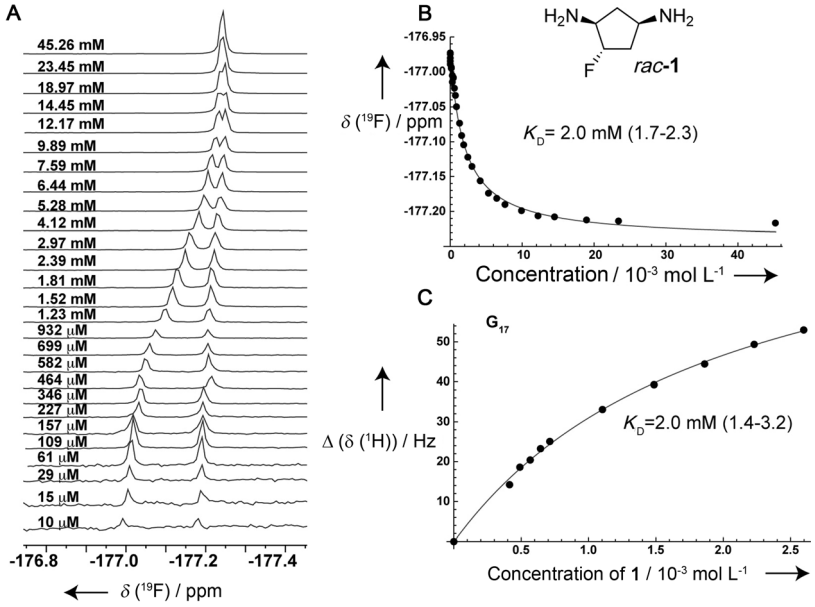
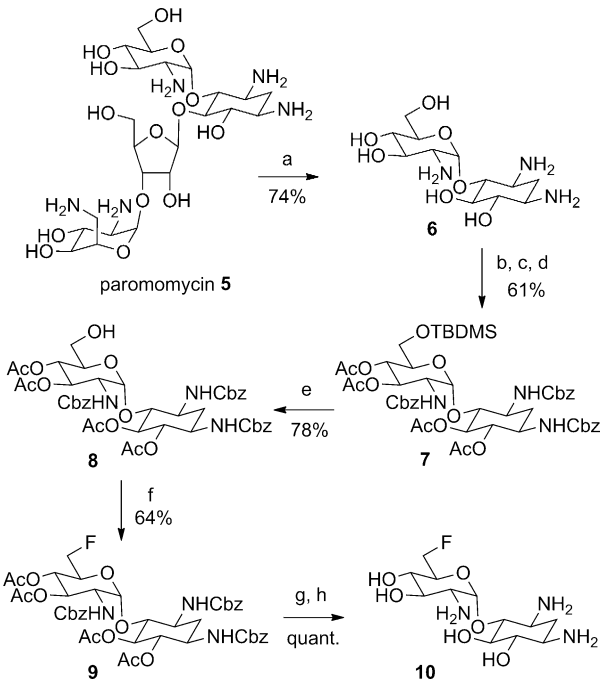


Figure 2. Measurement of the K_D between compound **1** and 16S23 RNA (0.3 mM) by ^{19}F NMR spectroscopy in KPO_4 buffer pH 6.5 containing 50 mM KCl at 293 K. A) ^{19}F chemical shift for increasing concentrations of probe. B) K_D determination based on the ^{19}F NMR chemical-shift variation (observation of the probe). C) K_D determination based on ^1H NMR chemical-shift variation of the 16S23 G_{17} base (observation of the target).

range of 120 μM, 50 μM, and 35 μM, respectively. The ranking of the K_D values of these molecules is thus in agreement with published data.[10] However, we would have expected a greater difference in the K_D values of neomycin and neamine. The inability of compound **1** to accurately measure this difference might be from too great a difference in dissociation constants between reporter **1** and neomycin.[14]

For the purpose of ranking higher affinity ligands, we prepared a novel fluorinated reporter that could bind in a more selective and efficient manner. Compound **10** was therefore prepared from paromomycin **5** (Scheme 1). This compound showed a sixfold lower dissociation constant for the 16S23 RNA than compound **1**, as determined by ^1H NMR titration ($K_D = 300$ μM).[15] However, its improved K_D led to a significant broadening of its ^{19}F NMR signal in the presence of 16S23 RNA, as a result of an intermediate exchange regime. Fortunately, a sharp signal could be recovered by increasing the ionic strength of the buffer to 300 mM KCl.[15] Working at a high salt concentration has two interesting consequences: 1) it decreases the K_D of the reporter for the RNA target, and the interaction is back to a fast regime exchange on NMR timescale; 2) the electrostatic part of the binding interaction is lowered, and thus the promiscuous binding that often occurs with an RNA target is limited.[16] Using this reporter **10** under these conditions, we estimated a K_D of 60 μM for neomycin, 90 μM for paromomycine and 250 μM for neamine (Figure 4). This ranking is in agreement with ones determined by other techniques.[10] Interestingly, raising the salt concentration increases K_D values of 16S23 ligands, and tends to increase the differences between good ligands, thus allowing a more precise ranking.[17] Thus, neamime and neomycin, initially ranked very close to each other using reporter **1**, were better differentiated using a tighter 16S23 RNA binder like compound **10** at a high-salt concentration.[7] Thus, two fluorinated probes that present a broad spectrum of interaction with RNA targets can be used to design specific ligands towards an RNA target. In a fragment-based approach, compound **1** is a better suited molecule to perform competition experiments whereas, in optimization assays, compound **10** will be more efficient.

We then investigated the use of fluorinated compounds to monitor RNA conformational changes upon ligand binding (principle from Figure 1B). As a case study, we investigated the interaction of neomycin with a known neomycin aptamer.[18] This aptamer is particularly interesting since it adopts an unbound "on" conformation and a bound "off" conformation in the presence of its ligand.[18] This conformational switch can be visualized by ^1H NMR, (Figure 5A,D). Ligand binding induces extensive structural changes, as indicated by the appearance of new imino protons upon addition of neomycin (Figure 5A).[19] We first analyzed the interaction between the aptamer and com-



Scheme 1. Reaction conditions: a) HCl, MeOH, heated to reflux, overnight; b) benzylchloroformate (CbzCl), Na_2CO_3, toluene/water/acetone, −5 °C to RT, 4 h; c) *tert*-butyldimethylsilyl chloride (TBDMSCl), 4-dimethylaminopyridine (DMAP), Et_3N, dimethylformamide (DMF), −5 °C, 1 h; d) Ac_2O, DMAP, pyridine, RT, 1 day; e) HF/pyridine, tetrahydrofuran (THF), RT, 4 h; f) diethylaminosulfur trifluoride (DAST), dry dichloroethane, −5 °C to RT, 5 h; g) NaOMe, MeOH/THF/dioxane, RT, 3 h; h) H_2, Pd/C, dioxane/MeOH/water, RT, 2 days.

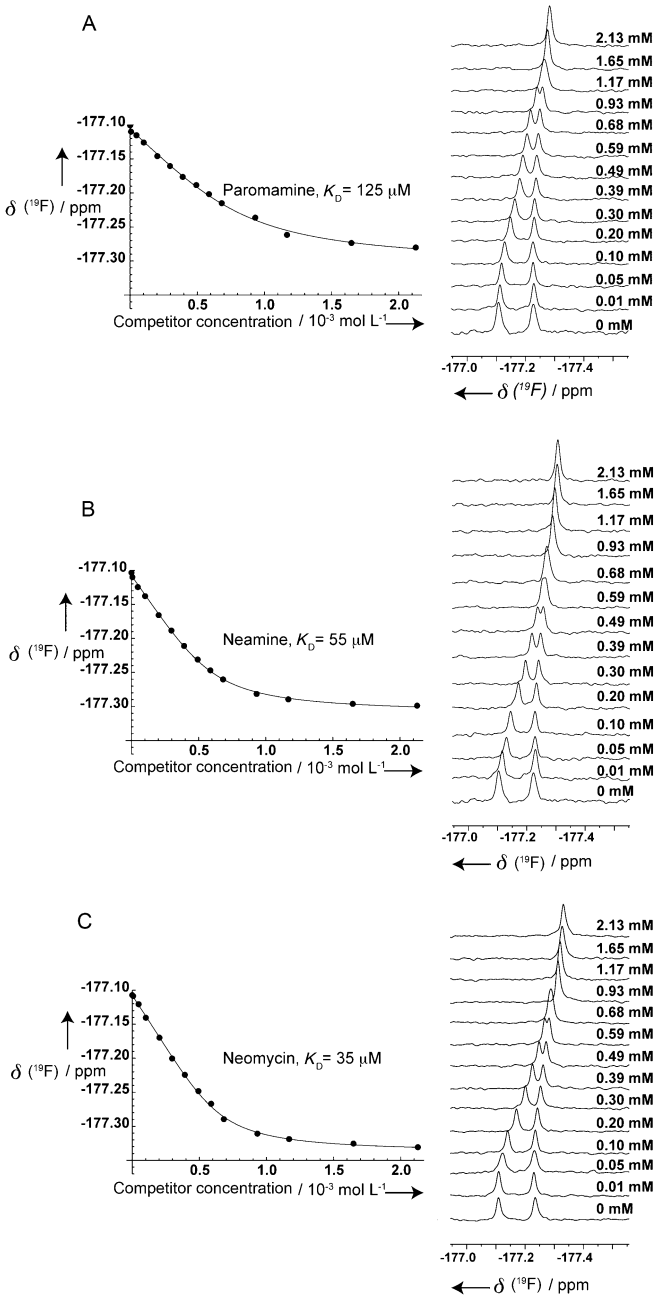


Figure 3. Competition experiments using compound **1** as the fluorinated probe. Right: ^{19}F NMR spectra of compound **1** (0.7 mM) mixed with 16S23 RNA (0.3 mM) in KPO_4 buffer pH 6.5 containing 50 mM KCl at 293 K with increasing concentrations of A) paromamine, B) neamine, and C) neomycin.

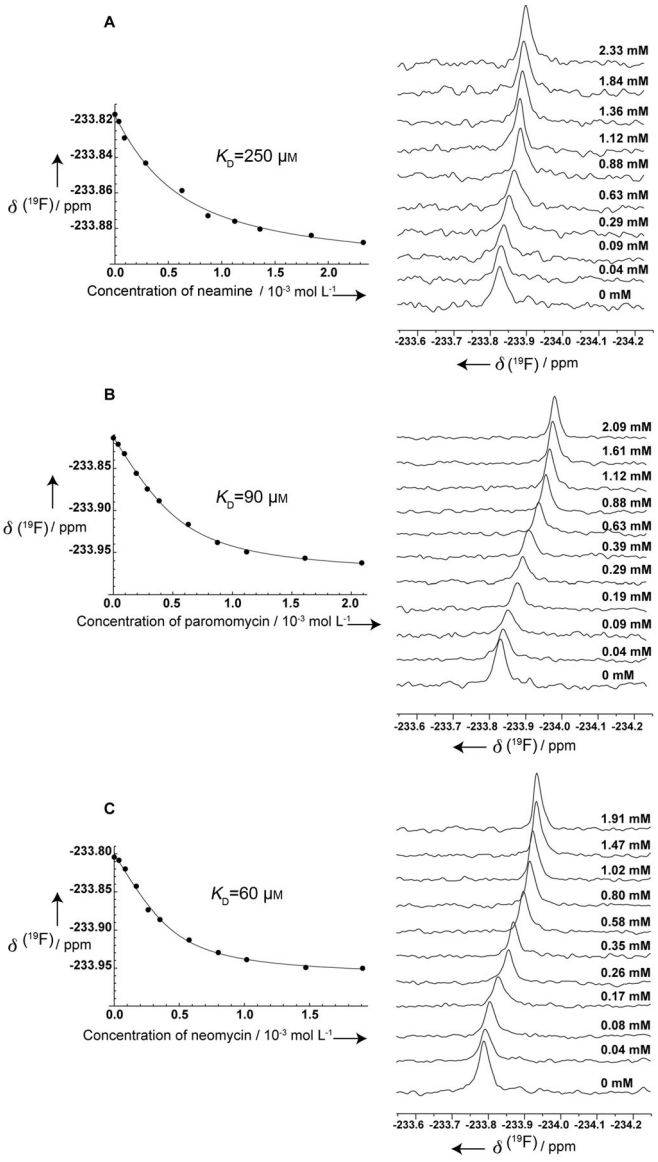


Figure 4. Competition experiments with **10** as the fluorinated probe: ^{19}F NMR spectra of compound **10** (0.7 mM) mixed with 16S23 RNA (0.3 mM) in KPO_4 buffer pH 6.5, containing 300 mM KCL, at 293 K, with increasing concentrations of A) neamine, B) paromomycin, and C) neomycin.

pounds **1** and **10** (Figure 5). Both probes bind to the aptamer but with different outcomes (Figure 5 B,C). Compound **1** binding does not alter the conformational equilibrium of the RNA target (Figure 5 C). Only chemical-shift variations are observed upon addition of compound **1** leading to a K_D value in the millimolar range. This result supports the idea that this external probe will induce only very limited bias in the study of the interaction between ligands and the RNA target. On the contrary, the higher affinity of compound **10** for the aptamer is illustrated by a complete disappearance of

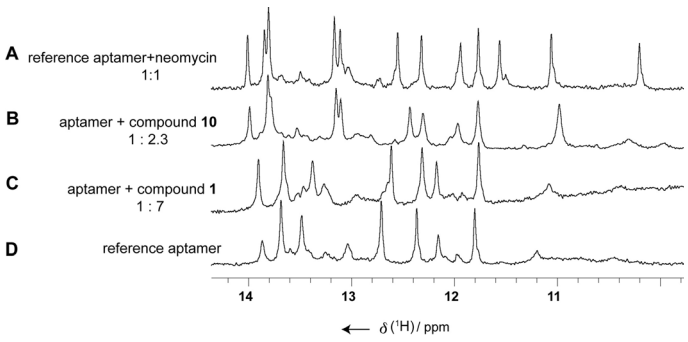


Figure 5. ^1H NMR spectra of neomycin aptamer (0.2 mM) in KPO_4 buffer pH 6.5 containing 50 mM KCl, at 293 K with A) one equivalent of neomycin, B) compound **10** (0.46 mM), C) compound **1** (1.4 mM), and D) alone.

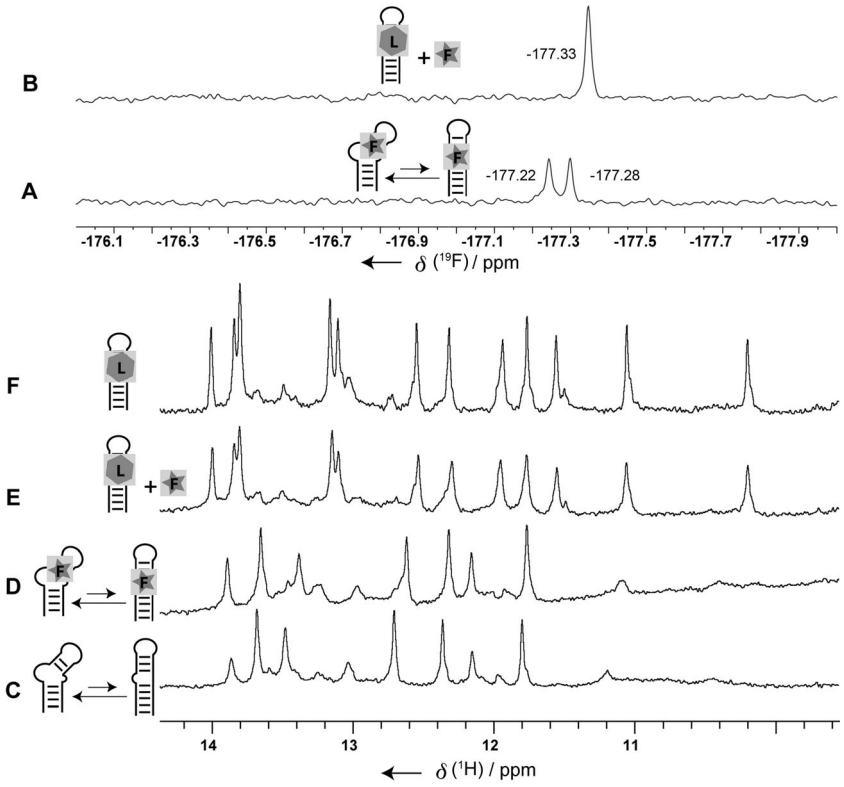


Figure 6. Dynamic folding of neomycin aptamer monitored by NMR spectroscopy using the fluorinated reporter **1**. ^{19}F NMR spectra of neomycin aptamer (0.3 mM) in a phosphate buffer (10 mM, pH 6.5) with 50 mM KCl and A) compound **1** (0.7 mM), B) compound **1** (0.7 mM) and neomycin (0.45 mM). ^{1}H NMR spectra of neomycin aptamer (0.3 mM) in a phosphate buffer (10 mM, pH 6.5) with 50 mM KCl, C) alone, D) compound **1** (0.7 mM), E) compound **1** (0.7 mM) and neomycin (0.45 mM), or F) neomycin (0.3 mM).

its ^{19}F NMR signal upon binding, as a result of an intermediate exchange regime, and the folding of the aptamer into a nearly bound conformation (Figure 5B). These experiments clearly show that a reporter with high affinity for the RNA target should be avoided for monitoring conformationally labile RNA such as riboswitches, since it can dramatically alter their native conformational equilibrium.

Interestingly, the ^{19}F NMR signal of compound **1** is detectable upon binding to the neomycin aptamer (Figure 6A) and shows signal splitting owing to the binding of each compound **1** enantiomer to the aptamer.[8] A complete ^{19}F NMR signal coalescence to the ^{19}F signal of the free compound **1** was observed upon addition of neomycin to the mixture, which shows that the fluorinated probe was fully displaced (Figure 6B). Moreover, ^{1}H NMR spectroscopy revealed a profound change in the aptamer structure that now shows a neomycin-bound conformation (Figure 6E). These experiments demonstrate that the competitive displacement of a small fluorinated binder can be used to easily monitor the conformational capture of a bi-stable RNA by its ligand.

In conclusion, we have shown that small fluorinated reporters are useful for monitoring the binding of unlabeled RNA with unlabeled ligands. The observation of ^{19}F NMR chemical-shift variations can be used to qualitatively rank different binders for a structured RNA, such as the 16S23 RNA. Chemical modification of the reporter and tuning of experimental conditions enable the observation of ligands with different binding strengths. Fluorinated reporters can also be used to monitor the conformational capture of a bi-stable RNA upon binding. In this case, a reporter with a low affinity to the target behaves as a neutral partner and does not influence the equilibrium between the bound and unbound states, in contrast to a higher-affinity binder. The absence of any target or ligand labeling step, the 100% natural abundance of the ^{19}F nucleus, the bio-orthogonality of the carbon–fluorine bond, as well as the high ^{19}F NMR sensitivity make this method very attractive from a practical standpoint.[20] We believe that ^{19}F NMR spectroscopy competitive screening will be a simple and efficient method for the discovery of new RNA binders.

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[20] All the ^{19}F NMR spectroscopy experiments have been conducted on a 300 MHz (H value) spectrometer with a standard QNP probe. As **1** has been shown to bind to several structurally different RNAs, we believe that it should be an excellent tool to investigate many other RNAs.

