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PROTOCOL

NMR for non-experts; a practical guide for applying NMR methods in studies of aptamer-ligand interactions

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ABSTRACT

Nuclear Magnetic Resonance (NMR) Spectroscopy is a powerful technique for studying aptamer structure and function. Important information about ligand binding and how binding affects aptamer structure can be obtained relatively quickly using ^{1}H NMR spectra focused on the imino resonances. Here we summarise some practical information for preparing aptamer samples for NMR analysis, designing experiments, and interpreting results, with an emphasis on aptamer-small molecule interactions.

KEYWORDS: Aptamers, Nuclear Magnetic Resonance Spectroscopy, folding, small molecule interactions

INTRODUCTION

Aptamers are nucleic acid strands that bind to specific targets. Aptamers exist in nature as a part of riboswitches (Serganov and Nudler, 2013) and can also be selected in the laboratory setting, often for biosensor applications (Wu et al, 2019). There are a host of techniques available to study the affinity and binding kinetics of aptamer-ligand interactions including fluorescence spectroscopy, isothermal titration calorimetry (ITC), surface plasmon resonance (SPR) and microscale thermophoresis. There are fewer methods to study aptamer structure with X-ray crystallography and Nuclear Magnetic Resonance (NMR) spectroscopy by far the most common. Both X-ray and NMR methods can be used to determine the three-dimensional structure of an aptamer, both free and ligand-bound. However, NMR spectroscopy can be used to determine more than the 3D structure of an aptamer. NMR can also be used to provide insights into the location of ligand-binding sites, study structural changes in an aptamer or regions of the aptamer with ligand binding and to also look at the stability and dynamics of an aptamer.

In solution NMR spectroscopy, the sample studied is placed in a strong magnetic field that enables the spin ½ nuclei present in nucleic acids (^{1}H, ^{13}C, ^{15}N and ^{31}P) to be studied. The nuclei will resonate at a characteristic frequency depending on chemical identity and local environment. NMR is a non-destructive technique allowing for multiple experiments to be performed on the same sample under different conditions such as temperature, the amount of ligand present in the sample, and/or different the buffer conditions.

This protocol will cover the basics on performing NMR experiments as it pertains to studying aptamers. Topics such as sample preparation, buffer selection, and performing experiment will be covered. The experiments presented here rely on obtaining information about ligand binding and aptamer stability by following the peaks of imino protons in 1D ^{1}H experiments. The imino protons are so useful because their signals are located downfield (at higher ppm values), well separated from the signals of the rest of the protons found in nucleic acids (Figure 1). Imino proton signals generally only show up when hydrogen bonded, such as in a base pair. The position of an imino proton signal in an NMR spectrum is dictated by its chemical identity and environment. Imino protons in Watson-Crick base-pairs are the most downfield, showing up around 12-14ppm with guanine iminos around 12-13ppm and iminos from thymine or uracil around 13-14ppm. Non-Watson-Crick base pairs are generally found upfield of the Watson-Crick base pairs in the 10-12ppm range. To provide the most useful information the identity of the imino resonances should be determined. This can be done by analysis of a 2D NOESY experiment acquired in H_2O (Wüthrich, 1986).

MATERIALS AND METHODS

NMR Instrument

NMR instruments are available with fields ranging up to 1200MHz. The higher the field of the instrument, the greater the resolution and sensitivity. Data shown in this paper were acquired on a 600MHz Bruker Avance Spectrometer. For the type of experiments discussed in this manuscript a simple 2-channel instrument with a room temperature probe is sufficient. The number of scans acquired depends on the sample concentration, but ranges from 32 to 512 scans. The typical recycle delay used was 1.2sec, but values up to 2sec are commonly used. We note that both Bruker and Varian NMR instruments come with stock experiments, such as the 1-1 echo sequence, that are particularly well-suited for detecting imino signals.

Buffer Materials

Standard chemicals needed to prepare the desired sample environments. Deuterated forms of reagents may also be required for buffer preparation.

Aptamers

DNA can be purchased from a manufacturer with standard desalting purification. Other levels of purification can be used, but we have found this unnecessary. Additional purification is typically not need but buffer exchange of the sample upon arrival is required. RNA samples can be purchased or produced by *in vitro* methods (Milligan and Uhlenbeck, 1989).

Ligand

The ligand for the aptamer can be obtained as normal.

NMR Tubes

Standard 5mm NMR tubes can be purchased from most chemical supply companies.

Sample Preparation

The first step in NMR experiments is to prepare the aptamer sample. NMR samples generally require 500-600µL of liquid placed in a standard 5mm pyrex NMR tube. For DNA aptamers, sample is typically not limiting due to the ease of purchasing material and the high solubility of DNA. For typical 1D NMR experiments the aptamer concentration should be ~500µM, but can go down to 200µM, if required. For 2D experiments, a 0.8mM sample is typically needed, though higher is preferable. If sample material is limited and a choice between volume and concentration must be made, concentration should be the variable favored, as the signal intensity is directly proportional to the concentration of the sample. For NMR experiments, we typically purchase 2 to 4 samples of a 1µmole scale DNA syntheses.

DNA samples typically need only be buffer exchanged after purchase to remove any salts and reagents left over from the synthesis. In our experience, other forms of purification, such as preparative gel electrophoresis, are not needed when nucleic acid samples are purchased. Upon arrival, samples are dissolved in purified filtered water from a system such as a Milli-Q unit and placed in an Amicon-type centrifugal filter. For aptamers in the 30-40 nucleotide range a 5ml tube with a molecular weight cut-off of 3,000MW is ideal. First, the sample should be exchanged four times against a 2M NaCl solution. This helps removes any leftover salts and reagents from synthesis. The sample can then be exchanged into the desired buffer.

Sample Considerations

There are three main considerations that should be taken into account when choosing a buffer for an NMR sample. Firstly, the amount of salt should be kept to a minimum. A high concentration of salt in the sample (greater than 75-100mM) can lower the signal to noise ratio of the sample, especially when using a cryogenic probe. An example of this can be seen in Figure 2. A good amount of salt in an NMR buffer is 20-50mM, this amount should not cause too much of a problem with the signal to noise ratio. More salt can be present in an NMR buffer if it is required for the aptamer to fold, be stable or to function.

The next consideration should be that many buffers used in molecular biology such as TRIS and HEPES contain protons. These protons will show up in your NMR sample and generally drown out the signals from the aptamer. If the buffer does contain proton-rich reagents, deuterated versions of these reagents should be used. These deuterated versions are usually ~99% deuterated, meaning that there will be a small signal from the non-deuterated protons, but this is unavoidable. A 10-20mM phosphate buffer is recommended for a working pH range of 6.2-8.2.

NMR buffers require 5-10% v/v $^{2}H_2O$ to allow for the NMR probe to lock onto the sample. This means when preparing the sample buffer an appropriate amount of $^{2}H_2O$ needs to be incorporated into the buffer. This should also be incorporated into your ligand solution, if applicable, to keep the NMR environment constant. In addition, some experiments will require the aptamer to be in a 100% $^{2}H_2O$ buffer, so that the signal from exchangeable protons will not be observed. Typically, experiments in H_2O are acquired first then the sample is lyophilized and taken up in $^{2}H_2O$ for experiment to be run in $^{2}H_2O$.

Finally, we note that NMR spectra where imino protons are to be detected are typically acquired at 5°C. Generally, the higher the temperature the data is acquired at, the lower the signal to noise ratio the iminos will have as they exchange with the bulk water faster at higher temperature. We will note that temperatures lower than 5°C should be used with caution as the freezing point of 100% $^{2}H_2O$ is 3.8°C.

Ligand preparation

As with selecting a proper NMR buffer, there are considerations that should be made when preparing the ligand sample. The smallest practical volume of ligand should be added in order to not dilute the sample too much and

reduce the NMR signal. Adding 25-50µl of ligand to the 500-600µl NMR sample is fine and will not reduce the signal to noise appreciably.

Some ligands are not soluble or only sparingly soluble in water, which means they may need to be dissolved in an organic co-solvent, such as DMSO. If this is needed, a deuterated form should be used, as that amount of protonated organic solvent will drown out the NMR signal from the aptamer. As with deuterated buffer reagents, there will be small NMR signals from residual protons in the deuterated organic solvent, but this is unavoidable. Small amounts of organic solvent (>5% v/v) should not cause much of a disruption of the aptamer sample (Lee et al, 2013). Figure 3 shows a titration of DMSO into an aptamer resulting in only small changes in the NMR spectrum. We note that even if a ligand needs a solution of 100% organic solvent like DMSO, adding 30µl to a 600µl NMR sample will only result in a 5% final solution. Before performing a titration of the aptamer and ligand in organic solvent, it may be worthwhile to do a titration of the aptamer with only the organic solvent, in order to see how the organic solvent affects the NMR signals.

Imino ^{1}H assignments

The 2D NOESY experiment allows for the imino proton resonances to be assigned so that in further experiments it is known which resonances correspond to which base pair. A NOESY experiment detects which protons are close in space, typically within 5 Å. In a standard B-form or A-form helix the imino proton in a base pair will show an NOE signal to the imino protons of the adjacent base pairs in the helix. Depending on the structure of the aptamer, this should allow for most of the imino protons in the spectrum to be assigned. An example of this can be seen in Figure 4.

Ligand binding titrations

A titration of the aptamer with ligand monitored by a series of 1D ^{1}H spectra can yield very useful information about the location of the binding site as well as if, and how, the structure of the free aptamer changes as ligand binds. Resonances can change chemical shift with ligand addition due to: (1) being close to the binding site and having their magnetic environment affected by presence of the ligand; or (2) due to structural change occurring with ligand binding, even if the structural change is away from the binding site. A key consideration when analyzing a titration is if there is a change in the number of imino protons observed in the free and bound spectra. If there is an increase or decrease in the number of imino protons observed, that can indicate that new base pairs are being formed or broken during binding and this is often an indication of structure being formed or

lost. If the number of imino peaks observed is the same free and bound, there can still be structural changes taking place, just without changing the number of base pairs present.

An example of a titration of an aptamer with a ligand is shown in Figure 5a where the MN4 cocaine-binding aptamer is titrated with amodiaquine (Slavkovic et al, 2018). In this titration, imino resonances change their position with ligand binding but the total number of resonances changes very little, if at all. Here, the resonances that change chemical shift are most likely close to the ligand-binding site of the aptamer.

An example of a titration of an aptamer where the number of imino protons increases with ligand binding is shown in Figure 5b. Here, the imino protons in one stem of the MN19 cocaine-binding aptamer are not observed in the free form and appear as ligand is added (Neves et al, 2010a; Neves et al, 2010b). Presumably, in the free form stem 1 is unfolded, dynamic or loosely folded in the free state and folds or rigidifies in the bound state.

Thermal stability

A series of 1D ^{1}H spectra acquired as temperature is varied, a thermomelt, tracks how peaks in an aptamer change and the aptamer unfolds as the temperature increases. Starting at a low temperature, multiple spectra are acquired, increasing the temperature after each spectrum to see how the resonances change. Starting the series at 5^{o}C and increasing the temperature of the sample by 5^{o}C each time is adequate. An example is shown in Figure 5c. If peaks disappear too quickly using the above scheme, starting the series at a lower temperature and using smaller temperature increments may be advantageous. An advantage of this technique over other techniques to monitor melting is that each imino proton can be tracked individually, allowing one to see if a global 2-state unfolding occurs or if different sections or helices of the aptamer unfold separately as the temperature increases. It is also possible that some peaks disappear before others due to them being more susceptible to exchange with water. As the temperature increases, the signal-to-noise ratio of the spectrum will decrease as the individual peaks broaden. We note that the temperature that resonances disappear in the imino NMR spectrum is typically lower than the melt temperature determined using in other methods. A temperature scan with and without ligand present is a useful way to confirm binding, as the bound form of the aptamer will generally melt at a higher temperature.

FURTHER READING

In this protocol we have only scratched the surface of NMR applications to studying aptamer-ligand interactions. For more in-depth reviews of NMR applications to studying nucleic acid dynamics and structures we refer the reader to other reviews (Dominguez et al, 2011; Adrian et al, 2012; Barnwal et al, 2017; Sakamoto, 2017; Choi et al, 2019; Marušič et al, 2019; Schnieders et al, 2020).

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

NMR: Nuclear **M**agnetic **R**esonance

DMSO: Dimethyl **S**ulf**o**xide

Tris: Tris(hydroxymethyl)aminomethane

HEPES: 4-(2-**h**ydroxy**e**thyl)-1-**p**eperazine**e**thane**s**ulfonic acid

NOE/NOESY: Nuclear **O**verhauser **e**ffect/ **N**uclear **O**verhauser **e**ffect **S**pectroscop**y**.

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FIGURE LEGENDS

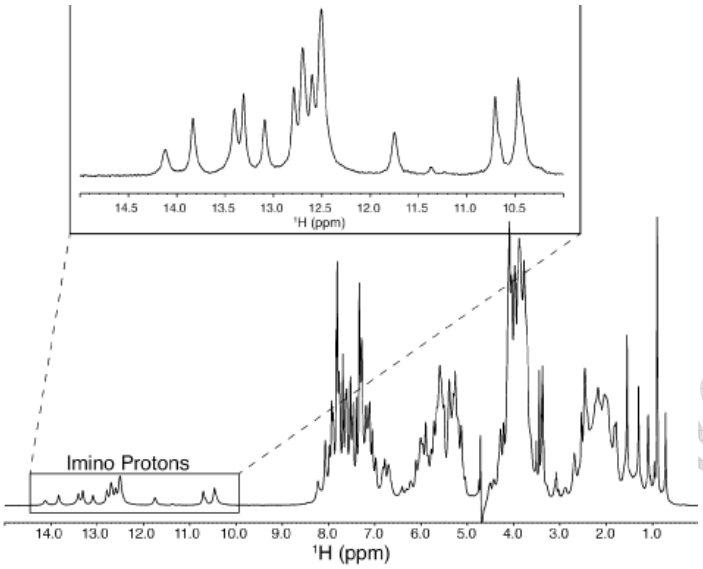

Figure 1. 1D ^{1}H MR spectrum of a DNA cocaine-binding aptamer bound to cocaine with an inset focused on the imino region.

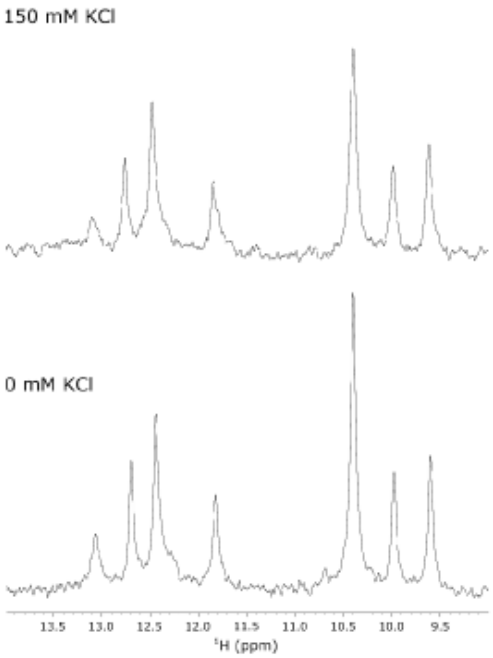

Figure 2. NMR spectra of a cocaine-binding aptamer bound to quinine, with either 0mM or 150mM KCl, illustrating the effect of salt on the NMR spectra. Samples are in 10mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) 10% $^{2}H_2O$, with an aptamer concentration of 360µM. The quinine:aptamer molar ratio is 1:1.

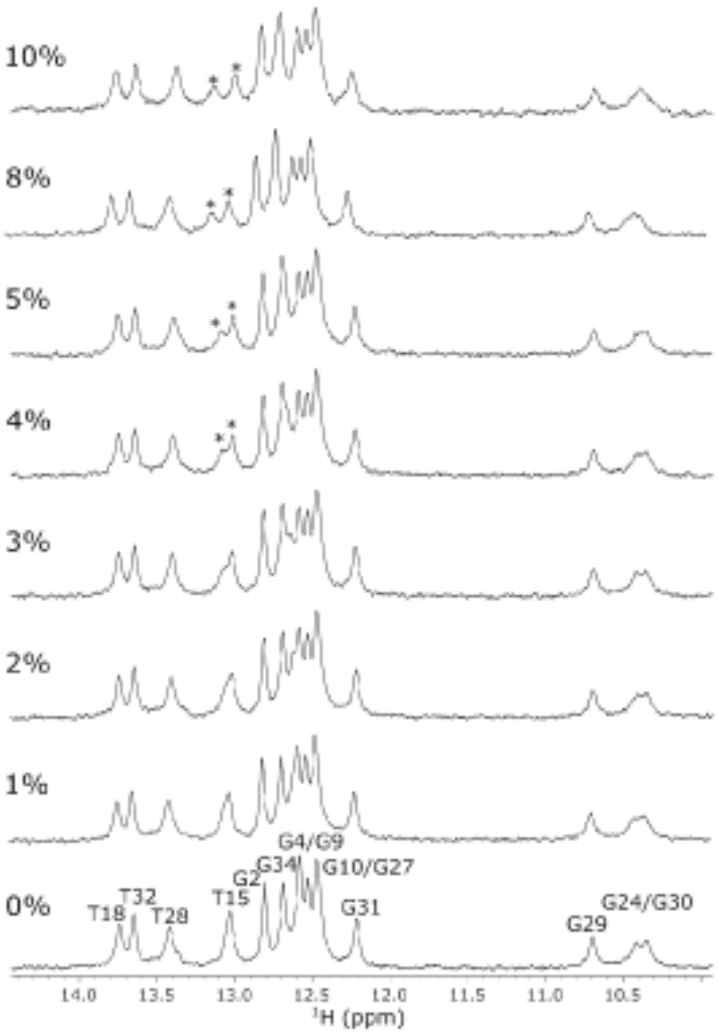

Figure 3. NMR spectra of an unbound cocaine-binding aptamer being titrated with DMSO. The percentage DMSO (v/v) is indicated. The DMSO does not have a large effect on the spectra until approximately 4% v/v. The asterisk denotes T15 splitting into 2 peaks possibly indicating that DMSO binds near this nucleotide. Titration takes place in 10mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) 10% ^2H_2O, with an aptamer concentration of 200µM.

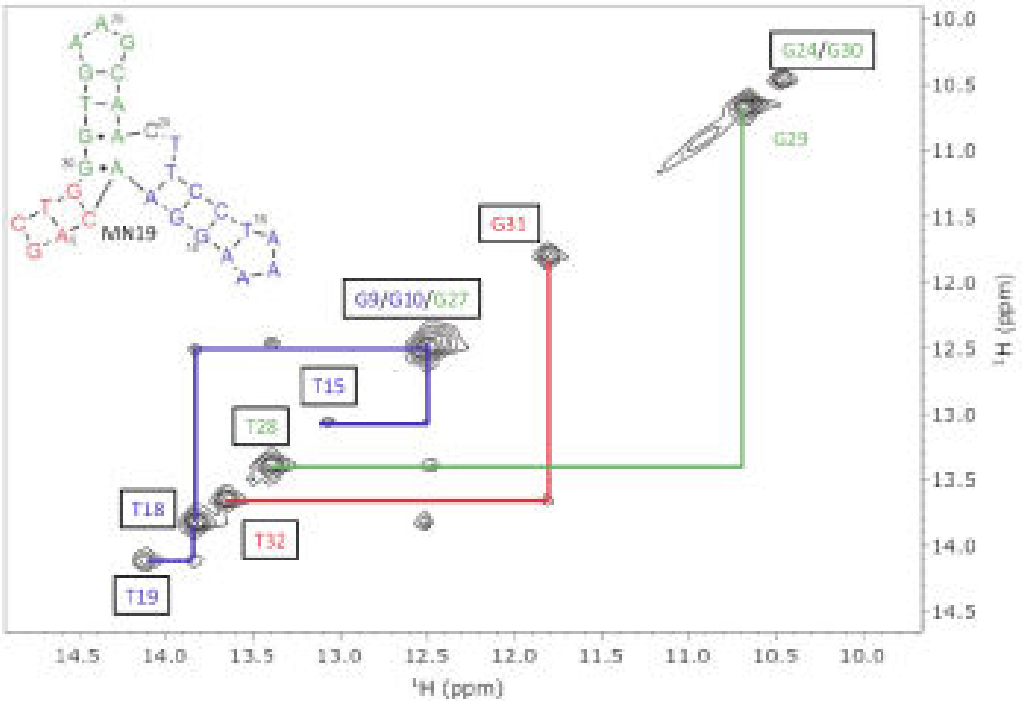

Figure 4. NOESY of a cocaine-binding aptamer (MN19) bound to cocaine. The secondary structure of the aptamer is shown in the top left, with the three stems being highlight in red (stem 1), blue (stem 2), and green (stem 3). The NOE steps are coloured to show which stem they represent in the aptamer. This experiment was conducted in 245mM KCl, 5mM KH_2PO_4/K_2HPO_4 (pH 6.8) 10% ^2H_2O, with an aptamer concentration of 1.5mM. The ligand:aptamer molar ratio is 1:1.

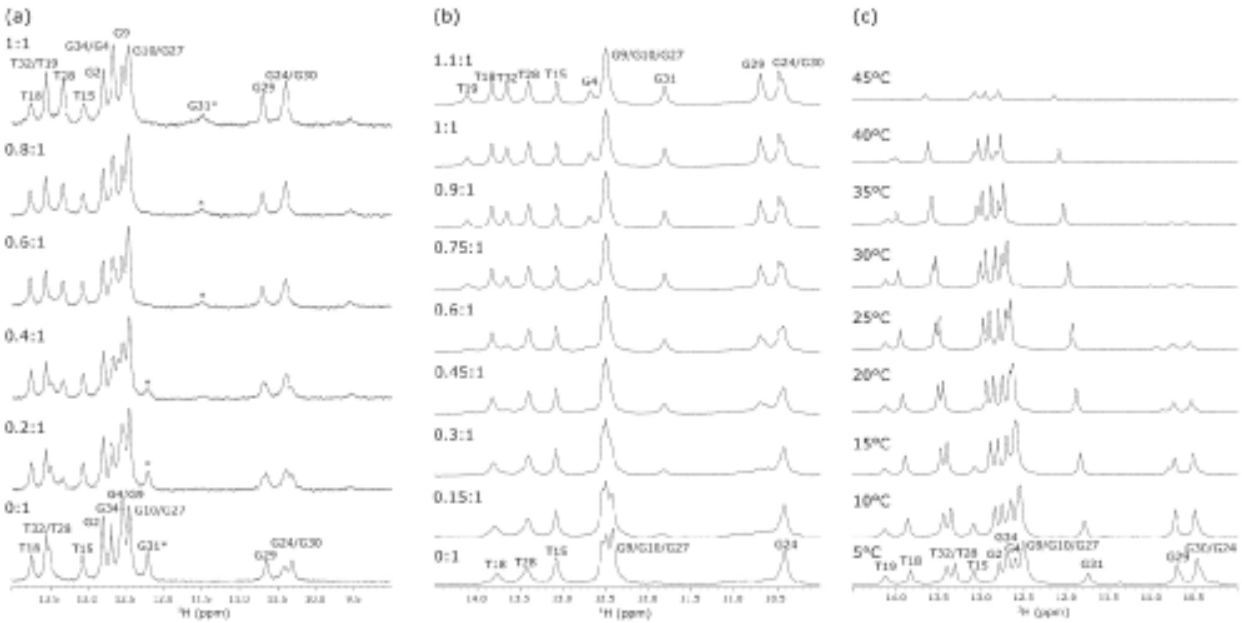

Figure 5. (a) Titration of a cocaine-binding aptamer with amodiaquine. G31, whose signal is particularly indicative of binding, is marked with an asterisk. Amodiaquine was dissolved in DMSO-d_6 (99.96%), and the concentration of DMSO at the 1:1 spectrum was about 2.5% (v/v). This titration was conducted in 10mM NaH_2PO_4/Na_2HPO_4 (pH 7.4) 10% ^2H_2O, with an aptamer concentration of 200µM. The molar ratios of ligand:aptamer are indicated. (b) Titration of a cocaine-binding aptamer (MN19) with cocaine. As the amount of cocaine present in the sample increases, more peaks become visible (G4, T19, G29, G30, G31, T32) and the peaks present previously become sharper and more defined. This titration was conducted in 245mM KCl, 5mM KH_2PO_4/K_2HPO_4 (pH 6.8) 10% ^2H_2O, with an aptamer concentration of 1.5mM. The molar ratios of ligand:aptamer are indicated. (c) Temperature scan of a cocaine-binding aptamer bound to cocaine at a 1:1 ratio. The sample was in 245mM KCl, 5mM KH_2PO_4/K_2HPO_4 (pH 6.8) 10% ^2H_2O, with an aptamer concentration of 1.0mM.