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PROTOCOL/METHOD

Isothermal titration calorimetry studies of aptamer-small molecule interactions: practicalities and pitfalls

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ABSTRACT

Isothermal titration calorimetry (ITC) is a powerful technique for studying binding interactions. From a single ITC experiment it is possible to quantify the affinity and thermodynamics of a binding event. Here, we outline an experimental approach for performing ITC experiments with a focus on aptamer-small molecule interactions. We also discuss some common problems that can be encountered and how to resolve these issues.

KEYWORDS: Aptamers, isothermal titration calorimetry, binding thermodynamics

INTRODUCTION

The term aptamer refers to a nucleic acid molecule that binds another molecule. Quantifying the binding parameters of an aptamer-ligand pair is an important part of characterizing the function of an aptamer. Isothermal titration calorimetry (ITC) is a versatile technique for performing binding studies, as it is easy to perform a series of experiments under many different solution conditions. ITC also benefits from being a non-destructive label-free technique that provides the stoichiometry of interaction and a complete set of the thermodynamic binding parameters: the equilibrium binding constant (K_a or K_d) and the change in enthalpy (ΔH) and entropy (ΔS) of binding. These benefits do come with the disadvantages that ITC uses a lot of sample compared with many spectroscopic methods and it has a low throughput of 2–4 runs being able to be performed per day.

In a typical ITC instrument (Figure 1a) two cells are placed in an adiabatic jacket. One of these cells is a reference cell while another is loaded with sample. These cells are maintained at a constant temperature (Figure 1a; $\Delta T_1 = 0$). During an experiment, ligand is titrated into the sample cell in known aliquots (Figure 1b). In an exothermic binding event, the temperature in the sample cell increases upon addition of ligand causing a decrease of power to the heater around the sample cell that maintains the reference and sample cells at an identical temperature. As a result, the raw experimental data is comprised of a series of negative spikes, where every spike corresponds to one ligand injection (Figure 1c, top). For an endothermic reaction, the opposite occurs and a positive peak results. These ligand injections are performed repeatedly and upon the ligand binding sites in the aptamer becoming saturated, the heat signal decreases until only the heat of dilution of the ligand is observed. Integration of this power supplied per unit time yields the heat per mole of injectant with respect to the molar ratio (Figure 1c, bottom). Data fitting is performed on the integrated heats to quantify the binding parameters.

In this protocol we outline how to perform a typical ITC experiment with an emphasis on studying aptamer-small molecule interactions. We go over how to select the typical experimental parameters and how to troubleshoot common problems to ensure you acquire good-quality ITC data for both aptamer and non-aptamer projects.

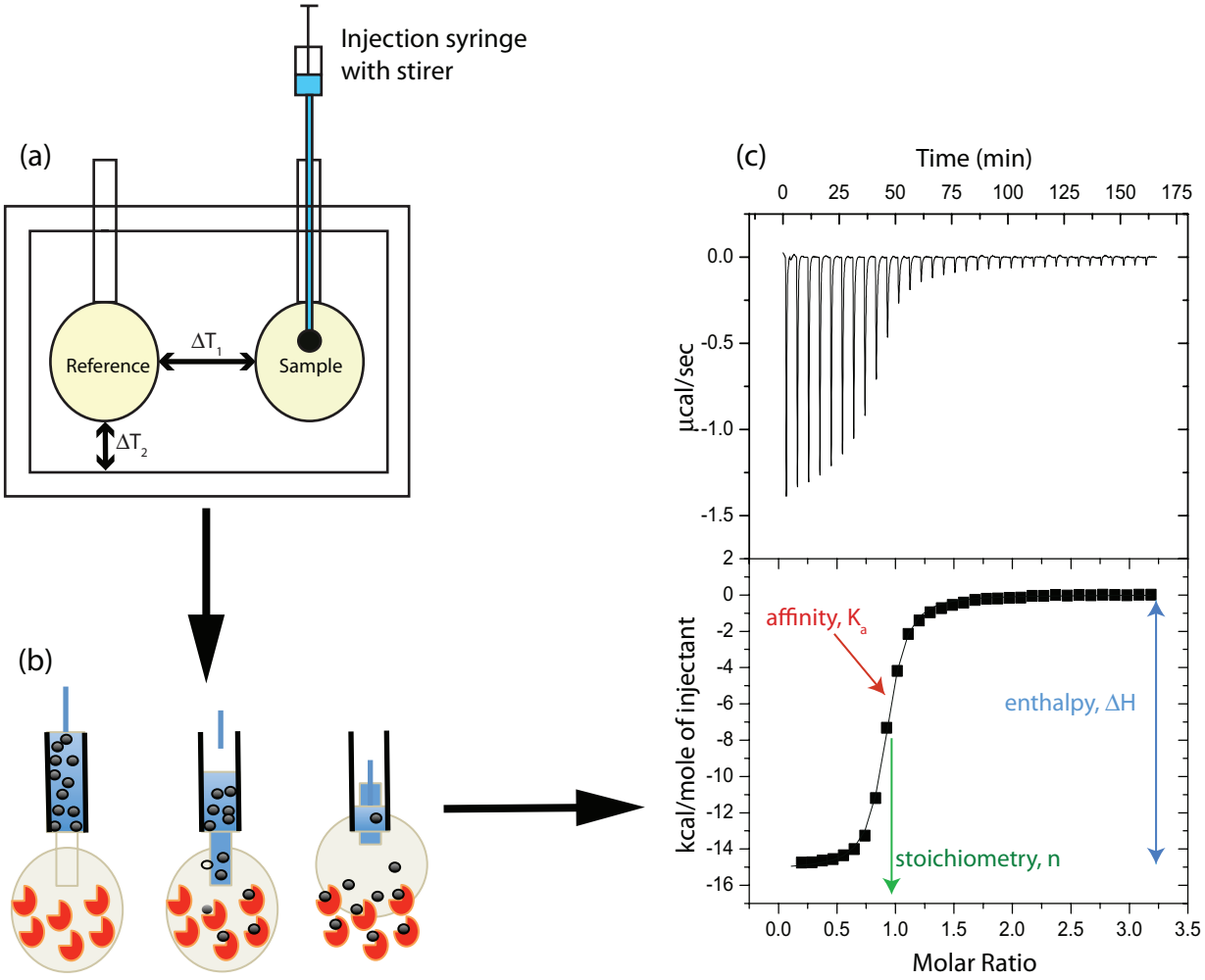

Figure 1. (a) Diagram of a typical ITC instrument. ΔT1 corresponds to the temperature between the two cells. The difference between cell and adiabatic jacket is referred to as ΔT2. Both ΔT1 and ΔT2 are zero during an experiment. (b) Depiction of binding during the gradual injection of ligand into the ITC cell containing aptamer. (c) Sample thermogram showing interaction of a cocaine-binding DNA aptamer with its ligand (Slavkovic et al, 2015). On the top is the heat from each injection with respect to time with the heat of dilution subtracted. On the bottom are the integrated heats for each injection (filled squares) fitted to a 1:1 binding model (solid line). Data is for the titration of 0.312mM quinine into 0.02mM MN4 aptamer at 15°C in 20mM TRIS (pH 7.4), 140mM NaCl, 5mM KCl. The injection enthalpy was plotted against the ligand-aptamer molar ratio and fit to 1:1 binding model to obtain a Kd value of 0.20±0.05µM, ΔH of -14±1kcal/mol and an n value of 0.98.

MATERIALS AND METHODS

ITC instrument: There are two manufacturers (MicroCal (Malvern) and TA Instruments) of commonly used instruments. In this protocol all methodology refers to the VP-ITC from MicroCal. A ThermoVac unit for degassing typically comes with the ITC instrument.
Buffer materials: Chemicals to prepare the desired sample conditions.
Aptamer: DNA and RNA can be purchased commercially with standard desalting. Additional purification can be done but typically isn't necessary.
Ligand.
Contrad 70: used for cleaning the ITC sample cell.

Sample Preparation

There are several aspects to be considered in sample preparation for ITC analysis: (1) optimal aptamer and ligand concentrations; (2) choice of buffer; (3) organic solvents (optional, if needed). According to the VP-ITC manual (MicroCal), the optimal aptamer concentration should be 10 to 50 times the anticipated K_d value. A good starting concentration range for aptamer-small molecule interactions is 10–20µM. These concentrations may be adjusted depending on the binding enthalpy of the interaction between aptamer and ligand, as well as the binding constant. For the ligand, its concentration should be 15–20 times the concentration of the aptamer in the sample cell. If the ligand concentration is much larger than the aptamer concentration, saturation will occur too soon producing a steep curve that saturates quickly and will yield an inaccurate fit. The VP–ITC instrument requires a sample volume of ~2ml of aptamer even though the cell measures 1.44ml in volume. The injection syringe volume is 290µl, but 600µl of ligand should be prepared. These larger volumes are necessary in order to fill both sample cell and syringe in a bubble-free manner. Other ITC instruments have different cell and syringe volumes, this should be checked prior to sample preparation.

When choosing these initial concentration parameters it also useful to consider the c–value (eqn. 1). This unitless parameter defines the shape of the binding curve (Figure 2)

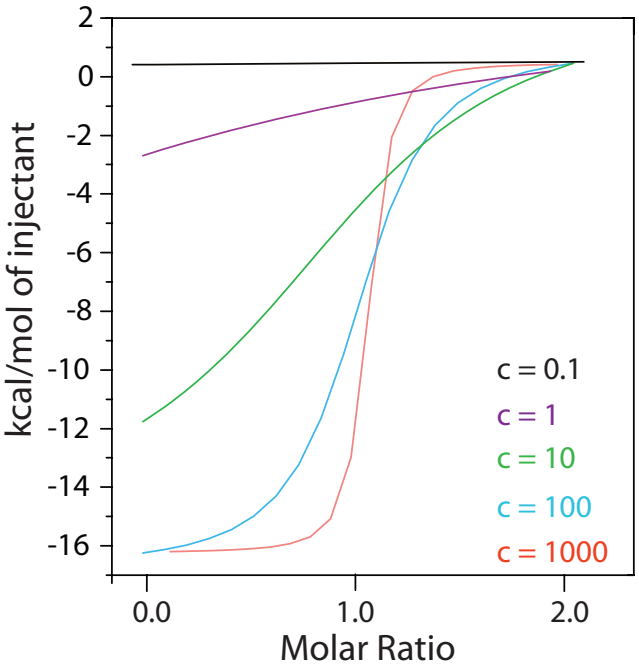

Figure 2. Illustration of the effect of the c-value on the shape of the titration curve.

and is the product of the binding constant K_a, total aptamer concentration at the start of experiment M_{tot} and the stoichiometry parameter, n:

$$c = K_a M_{tot} n \quad (1)$$

An optimal c-value lies between 10 and 100, though there is discussion in the literature about this (Turnbull and Daranas, 2003; Tellinghuisen, 2008; Broecker et al, 2011; Tellinghuisen, 2016). High c-values (over 500) give binding curves too steep to accurately determine affinity, although stoichiometry and enthalpy are well determined. Data acquired at c-values below 10 results in a shallow titration curve where all three parameters (n, K_a and ΔH) are poorly determined. These three fitting parameters are directly proportional to ligand concentration accuracy, while aptamer concentration accuracy only affects stoichiometry value.

In preparing the aptamer for ITC analysis we have had excellent experience in purchasing DNA samples with the standard desalting provided by the manufacturer. The DNA is dissolved in ~1ml ddH_2O for a 1 micromole scale synthesis and then exchanged 3 times against 1M NaCl using Amicon-style concentrators to compete off any unwanted substances bound to the nucleic acid. The sample is then exchanged 4–6 times against ddH_2O and the final volume of this stock solution is ~0.25ml from a 1 micromole scale synthesis. The aptamer concentration is measured using UV spectroscopy and the known extinction coefficient. Typically, 10–30µl of the stock aptamer solution is diluted with the desired buffer to a 2ml final volume for analysis. Prior to loading, the DNA or RNA molecule should be heated in a 95°C water bath for 3–5min and cooled in an ice water bath for at least 10min before use to allow the aptamer to anneal in an intramolecular fashion. Aptamers where separate strands need to anneal should be left to cool to room temperature slowly. Once aptamer and ligand samples are prepared, they are degassed using a ThermoVac unit (or equivalent) for 5min. If samples are not adequately degassed, large spikes will be formed in the ITC baseline due to air bubble formation during the experiment.

Experimental Setup

In a typical ITC experiment, the ligand is titrated into the aptamer. Reversing the contents of the cell and syringe typically does not change the fitted parameters but can be done to ensure there are consistent results. If solubility of the ligand is a concern, the aptamer should be titrated into ligand, *i.e.* the least soluble material is used as titrate (in the cell) and the more soluble as titrant (in syringe). This is set at the start of an ITC experiment. Other instrumental parameters set by the user are number of injections, run temperature, reference power, initial delay, spacing, injection volumes and concentration of samples. Non-optimal or improper settings can have great impact on data quality.

The experimental temperature is determined by what the user desires and the stability of the aptamer. However, binding constants and the enthalpy of binding (ΔH) are temperature dependent. The temperature can be set between 2 to 80°C. Samples should be cooled prior to use to below the experimental temperature as it reduces ITC equilibration time. The total number of injections should be set to a total of 15–20 but this will also depend on each injection volume and the total volume of the syringe. If the aptamer and ligand interact in a 1:1 molar ratio, an individual injection volume should be chosen such that this ratio is achieved in 8–12 injections. The volume of each injection is kept constant at 7–12µl, with the exception of the first injection, which is usually set at 1–2µl. Data from the first injection is discarded prior to data analysis as it has been shown that it results from a volumetric error due to the backlash in the motorized screw that drives the syringe plunger (Mizoue and Tellinghuisen, 2004). Feedback mode/gain is another parameter that is set by the user. For a typical ITC binding experiment, it is set to be high as it provides the fastest response time. For studies involving kinetics, this parameter is set at low or none.

Spacing refers to the time between consecutive injections. The time should be large enough to allow the signal to return back to baseline. This delay depends on the size of the peak – higher sample concentration produces larger peaks, which requires longer time between injections. Usually 300sec spacing is sufficient, but in some cases a longer time is needed (Figure 3).

Reference power is the amount of power that is supplied to the heater in the reference cell. A typical setting is 25–30µcal/sec for exothermic reactions. Endothermic reactions require a lower reference power setting. Stirring speed is typically set at 300*rpm* for aqueous solutions. Faster speeds will increase baseline noise levels but it may be necessary if the sample is more viscous than water.

Measurement of the heat of dilution of ligand is an important aspect of any ITC experiment as large heats of dilution of ligand can sometimes mask binding. Depending on the ligand, there are two methods that can be used to perform this correction: external and internal heat of dilu-

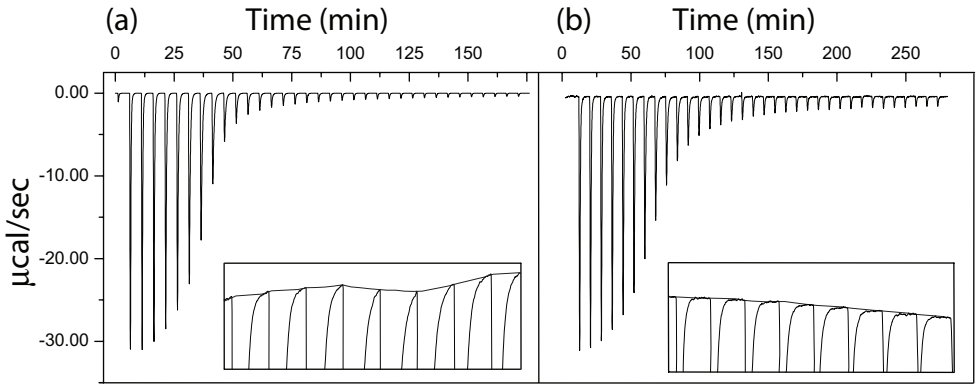

Figure 3. Adjustment of the time between injections. In (a) is an example of the interaction of the MN4 aptamer at 200 µM with quinine with the time between injections set at 300 s. The heat from an injection does not return to the baseline before the next injection takes place. In (b) the same experiment is performed with the spacing set at 480 s.

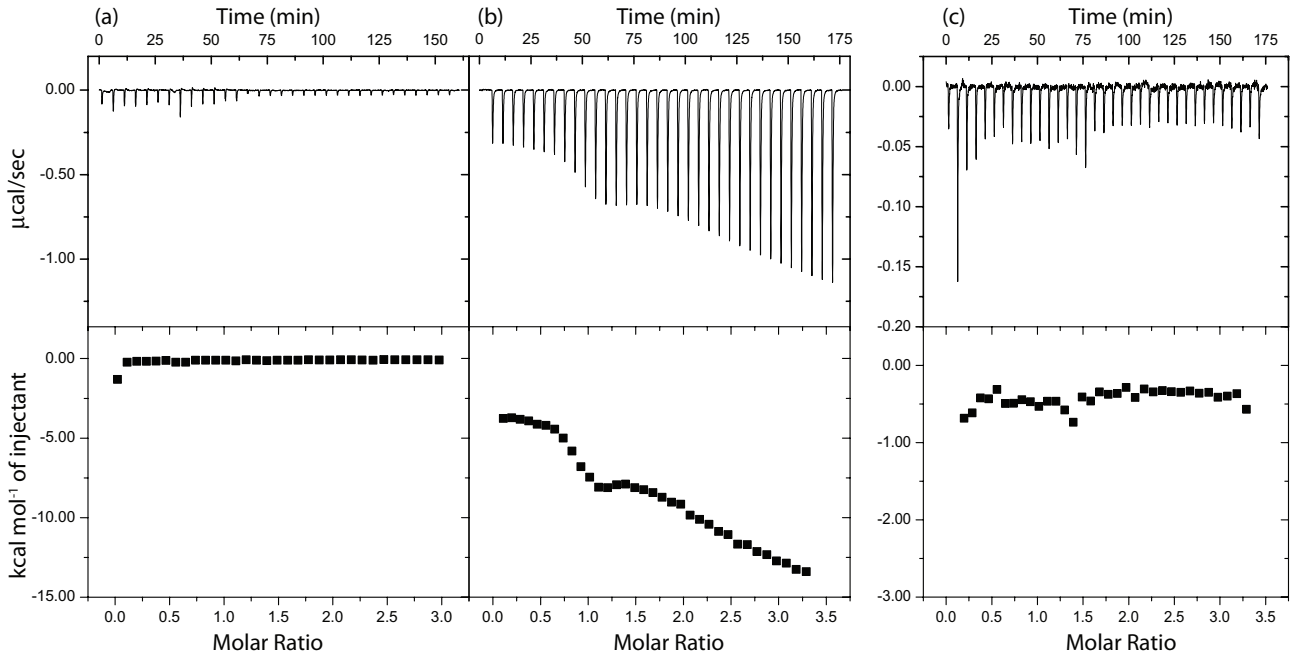

Figure 4. Examples of different possible heats of dilution. In (a) is a titration of quinine in buffer (20 mM TRIS (pH 7.4), 140 mM NaCl, 5 mM KCl) into a sample of the same buffer. This produces a small heat of dilution. The heat of dilution run in (b) is a sample of piperaquine into same buffer as in (a) but produces large and non-linear heat of dilution. (c) Titration of water into water is a good check of ITC cleanliness. A clean ITC sample cell and needle results in a very small heat signal with the integrated heats of ddH_2O titrated into ddH_2O being close to zero.

tion. External heat of dilution involves performing a separate experiment where ligand is titrated into buffer using the same conditions as the binding experiment (Figure 4a, 4b). An internal heat of dilution is obtained by extending the experiment by 5–10 injections after binding is complete (Figure 1c). Using a linear extrapolation of the heats of these injections a correction is applied to whole experiment. This method can be used only if heats of dilution are small and linear.

Cell Cleaning

A critical step in obtaining quality ITC data is cleaning both sample cell and injection syringe before loading samples. While nucleic acid aptamers are usually quite soluble and not prone to contaminating the cell (unlike protein samples), the ligands involved in binding may be less soluble and can accumulate on the sample cell wall. There are two types of cleaning: standard and stringent cleaning.

Standard cleaning is performed before every use. It involves flushing both sample cell and injector syringe with ddH_2O, washing with 200ml of 1% (v/v) Contrad 70 solution and flushing with about 1l of ddH_2O. Loading syringes should also be cleaned at this time. During an ITC experiment, raw data is plotted as differential power (DP) versus time. At the beginning of a titration, the user sets reference power, which is the amount of power continuously supplied to reference cell heater. If, for example, this value is set at 30µcal/sec, clean cell the baseline should be close to this value or 1–2µcal/sec lower. If the initial baseline is different from reference power, stringent cleaning is performed.

For stringent cleaning, the cell and injection syringe are flushed with 200ml of 5% (v/v) Contrad 70 then the cell is filled with the same 5% (v/v) Contrad 70 cleaning solution and left to soak for several hours at 60–70°C. The sample cell and syringe should then be exhaustively flushed with dd$H_2$0 (at least 1.5l).

Finally, before filling with samples, both the cell and syringe should be rinsed with buffer, and thoroughly emptied to avoid unwanted dilution. A useful check of instrument cleanliness is an ITC run of water titrated into water (Figure 4c). This run should produce very little heat and have the integrated heats very close to zero.

Data Analysis

Data analysis is typically performed with software supplied by the instrument manufacturer. For MicroCal instruments, the software employed is Origin. The software normalizes the heat of binding with respect to ligand concentration. It also sets the baseline and integrates each peak from the baseline. Most of the time the baseline is set appropriately automatically, but sometimes it needs to be set manually to exclude integration of artifacts. The heat of dilution should then be subtracted and finally the data fitted to the appropriate binding model. After performing these steps, the integrated data is used to determine n, K_a and ΔH by least squares minimization. Once these values are determined, it is simple to determine the Gibb's free energy (ΔG) and entropy (ΔS) according to the standard relationships (Equations 2 and 3):

$$\Delta G = -R\,T\,\ln K_a \quad (2)$$

$$\Delta G = \Delta H - T\Delta S \quad (3)$$

If an aptamer has one binding site, a simple one-site binding model is used, where n, K_a and ΔH are determined. In this case, n should be very close to 1. If n, for a 1:1 interaction, is closer to 0.8 or 1.2 then there is an issue with inaccurate concentrations of either ligand or aptamer. Provided the concentrations are correct, but the n value is very low it indicates a low c-value due to weak binding for the aptamer concentration used (Figure 2). In this case, the concentration of both aptamer and ligand should be increased to appropriately determine the three fitting parameters. Sometimes with small molecule-aptamer interactions the affinity of the interaction is quite weak and working at higher concentrations is not practical. In this case low-c ITC methods are available, provided the n value is known (Turnbull and Daranas, 2003; Tellinghuisen, 2008). There are often complementary experimental methods available that can determine the stoichiometry of the interaction, such as nuclear magnetic resonance (NMR) spectroscopy (Sakamoto, 2017). More complicated binding models such as two-site independent binding and sequential binding are also available but one should determine if their use is necessary based on the system studied (Table 1). Data fitting to these more complicated models is out of the scope of this article and the reader is referred to a number of excellent references (Freiburger et al, 2009; Freire et al, 2009).

If ligand binding is too tight, the affinity may be difficult to measure directly by ITC. In that case, it is possible to employ a displacement titration methodology where the aptamer is pre-bound to a weaker binding ligand and titration of the higher affinity ligand involves ligand exchange on the aptamer (Sigurskjold, 2000; Velazquez-Campoy and Freire, 2006). This method requires knowledge of the binding affinity of the weaker ligand in advance. For simple 1:1 interactions, Origin software has a built-in competitive binding model where the information for the weaker binding ligand (n, K_a and ΔH) can be entered and parameters for the tighter ligand determined.

Troubleshooting

The most common problems encountered when running ITC experiment are low binding heats, buffer mismatch, air bubbles, impurities and issues with the addition of organic solvents. If the observed binding enthalpy is low, the experiment can become heat limited. This can be overcome by increasing the aptamer concentration or injection volume as both will increase the heat detected per injection. In order to obtain best results, the first few injections should be at least 10µcal/injection and have an average of at least 5µcal/injection. This corresponds to a peak height of about 0.5µcal/sec. A low heat of binding produces an isotherm that is not well defined, as the heats of dilution are comparable to the binding heats. In this case, a series of experiments using the same conditions except at different temperatures can be performed. Increasing the experimental temperature typically increases the magnitude of the binding enthalpy. This increases the raw heats, which in turn gives greater signal to noise and a better-defined binding curve. The experiment can also be performed at a lower temperature. A measurement of binding enthalpy at several temperatures, in order to obtain the change in heat capacity (ΔC_p) can be used to predict binding enthalpy at a particular temperature.

An essential part of sample preparation is the buffer match between the aptamer and ligand. This is achieved by dialyz-

Table 1. Table listing the approaches that can be followed for different problems or situations encountered when analyzing ITC data.

Analysis Method	When to use the approach
One-site binding model	Sigmoidal shape curve that saturates at 1 or inflection point is at 1.
Two-site binding model	When a "u" or "v"-shaped binding curve observed.
Global fitting	To distinguish between cooperative and independent binding models when two-site binding observed.
Reverse Titration	When the ligand is poorly soluble and placed in the cell with the aptamer in the syringe. Also used to verify stoichiometry.
Low c-value	If binding stoichiometry is known, and ligand saturates the K_a but not ΔH can be extracted.
High c-value	Decrease aptamer and ligand concentrations used to extract K_a.

ing the aptamer in the buffer of choice and using the same buffer to prepare the stock solution of ligand. The same buffer must be used to dilute both aptamer and ligand to appropriate concentrations and fill the reference cell. The pH value of all components must be matched to avoid additional heats from pH mismatch. Difference in pH between aptamer, ligand and reference cell, gives large heat effect that show as artifacts or drift in baseline (Figure 5a). Another issue to consider is the ionization heats of different buffers. If binding involves protonation or deprotonation, binding enthalpy will vary widely between buffers (Fukada and Takahashi, 1998). It is best to choose a buffer, which give minimal additional heat contribution such as phosphate, citrate or acetate.

An additional problem that can be observed are large positive or negative spikes in the baseline (Figure 5b). The large positive spikes are usually indicative of air bubbles in the sample cell. The negative spikes can indicate that there are impurities in the sample cell such as leftover cleaning solution from insufficient rinsing during the cleaning process. Finally, Figure 5c is an example of a very poor baseline and results from the cell and syringe needing thorough cleaning.

Often, small molecule ligands require DMSO in order to solubilize. The final DMSO concentration should be minimal (up to 5% v/v) and added in the same amount to aptamer and ligand solution, as well as the reference cell. The heat

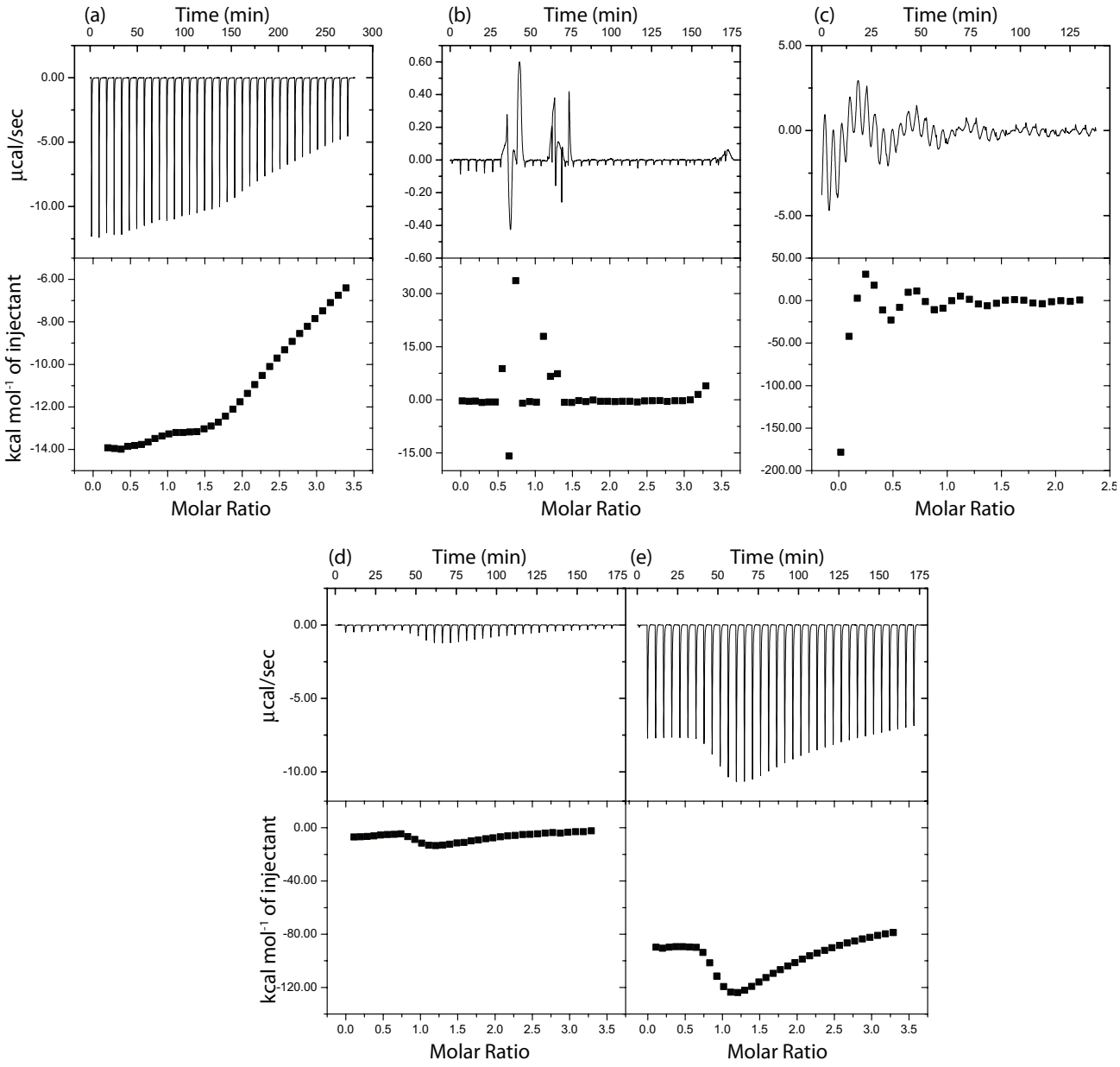

Figure 5. Examples of potential problems with ITC experiments. In (a) there is a pH mismatch between the aptamer in the sample cell and ligand injection syringe. In (b) there are large positive spikes indicating air bubbles in the sample cell. The negative spikes imply a possible impurity in the cell. In (c) there is an erroneous baseline. This indicates that both the sample cell and syringe require cleaning. Example of a mismatch in DMSO concentration between the cell and needle. In (d) is a titration of a ligand into a DNA aptamer where both aptamer and ligand are prepared in buffer containing 3% (v/v) DMSO. In (e) is the same titration except there is 6% (v/v) DMSO in the syringe and 3% (v/v) DMSO in the cell. This mismatch in DMSO concentration produces very large apparent heats of binding.

of dilution run should also contain identical amounts of DMSO. Any mismatch in DMSO content will result in large heats produced (Figure 5d,e). Other organic solvents can also be used to help solubilize ligands, but their concentration should be kept to a minimum. As the case with DMSO, these solvents should be added to both aptamer and ligand solutions and the reference cell at the same concentration. ITC cells are inert to most solutions, however strong acids must be avoided, as they will damage cells.

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COMPETING INTERESTS

None declared.

LIST OF ABBREVIATIONS

DMSO: **Dim**ethyl **s**ulf**o**xide
Δ**G**: Change in Gibb's free energy
Δ**H**: Change in enthalpy
Δ**S**: Change in entropy
ITC: **I**sothermal **t**itration **c**alorimetry
$\mathbf{K}_a$: Association equilibrium constant
$\mathbf{K}_d$: Dissociation equilibrium constant

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