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Optimizing isothermal titration calorimetry protocols for the study of 1:1 binding: Keeping it simple☆

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ABSTRACT

Background: Successful ITC experiments require conversion of cell reagent (titrand M) to product and production or consumption of heat. These conditions are quantified for 1:1 binding, M + X ⇔ MX.
Methods: Nonlinear least squares is used in error-propagation mode to predict the precisions with which the key quantities — binding constant K, reaction enthalpy $\Delta H°$, and stoichiometry number n — can be estimated over a wide range of the dimensionless quantity that governs isotherm shape, $c = K[M]_0$. The measurement precision σ_q is estimated from analysis of water–water blanks.
Results: When the product conversion exceeds 90%, the parameter relative standard errors are proportional to σ_q/q_{tot}, where the total heat $q_{tot} \approx \Delta H° [M]_0 V_0$. Specifically, $\sigma_K/K \times q_{tot}/\sigma_q \approx 25$ for $c = 10^{-3} - 10$, $\approx 11\ c^{1/3}$ for $c = 10 - 10^4$. For $c > 1$, n and $\Delta H°$ are more precise than K; this holds also at smaller c for the product $n \times \Delta H°$ and for $\Delta H°$ when n can be held fixed. Use of as few as 10 titrant injections can outperform the customary 20–40 while also improving productivity.
Conclusion: These principles are illustrated in experiment design using the program ITC-PLANNER15.
General significance: Simple quantitative guidelines replace the "c rules" that have dominated the literature for decades. This article is part of a Special Issue entitled Microcalorimetry in the BioSciences — Principles and Applications, edited by Fadi Bou-Abdallah.

Of all the methods for studying chemical binding in solution, only calorimetry can yield estimates of all three key thermodynamic properties for the binding process — $\Delta G°$, $\Delta H°$, and $\Delta S°$ — from experiments done at a single temperature. For this reason, isothermal titration calorimetry (ITC)[1] has become a preferred method for studying binding of moderate strength in a wide range of applications in a comparably wide number of disciplines. For illustration, a topic search of the Science Citation Index for "ITC OR isothermal titration calorimetry" turned up over 900 published papers in about 300 journals for the year 2014; these figures are about three times those for a similar search I did a decade ago [1].

In an ITC experiment for 1:1 binding, M + X ⇔ MX, one reagent (titrant X) is injected sequentially, with stirring, from a precision syringe into a cell containing the other reagent (titrand M) [2]. Commonly 10–40 such injections are programmed, spaced 4–10 min apart, to permit the instrument to return to baseline after each injection, in which there is incremental conversion of M to MX, producing the signal (usually compensation power) that is integrated to obtain the heat (Fig. 1).[2] By the conclusion of the experiment, the cell should contain an excess of titrant X large enough to ensure conversion of most of the original M to product. The data are analyzed by nonlinear least squares to obtain estimates of the binding constant K, $\Delta H°$, and the stoichiometry number n (= 1 for 1:1 binding when concentrations and cell volume are well known).

From this description, a successful ITC experiment requires (1) production or consumption of heat, and (2) conversion of the cell reagent to product. The worker planning the experiment must deal with a number of questions, most important of which is, "Will this work for my reaction?" If the answer is "yes," then one must decide on concentrations for reagents and the number m and volumes for the injections, keeping in mind practical considerations such as reagent expense, solubility, and aggregation. ITC experiment design has been addressed frequently in the literature, usually in the context of getting a suitable value for "c," a parameter (= $K[M]_0$, where $[M]_0$ is the initial titrand concentration in the cell) introduced in [2] that governs the shape of the titration profile (heat q vs. X:M ratio). In a recent contribution, I challenged these "c" prescriptions as too limiting and addressed a number of misconceptions

Abbreviations: ITC, isothermal titration calorimetry; LS, least squares; NLS, nonlinear least squares; SE, standard error; RSE, relative standard error; SD, standard derviation; MC, Monte Carlo.

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[2] This figure also illustrates a systematic error that can occur without warning, with devastating consequences. The great exothermicity of the reaction has driven the compensation power below zero, producing invalid data that appear to be normal.

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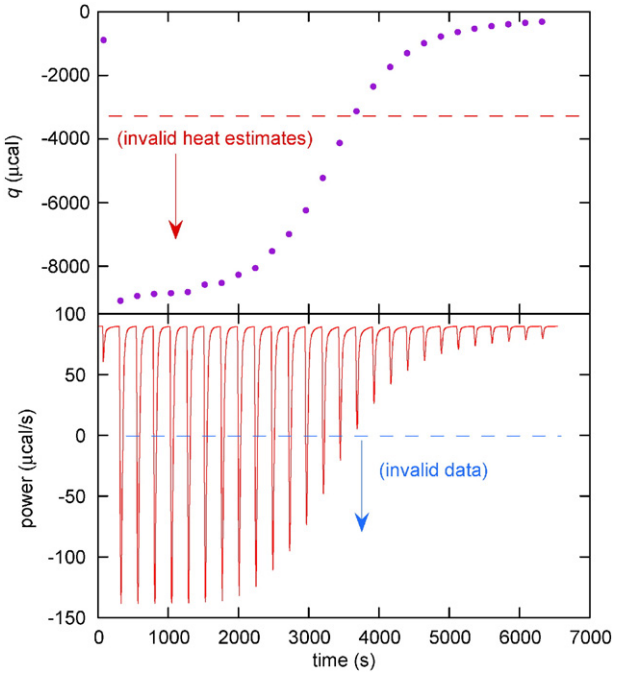

Fig. 1. Thermogram (lower) and integrated heats obtained for injecting 0.1 M $BaCl_2$ into 0.01 M 18-crown-6 ether on a MicroCal VP-ITC instrument with cell volume 1.37 ml. The program called for 1 2-µl and 26 9-µl injections. Note that most of the peaks drop below zero compensation power, making these data invalid.

that have persisted over the years [3]. However, many of the latter points are of minor practical significance, and in this work I focus on just the two essentials enumerated above: getting enough heat and sufficient conversion of titrand to product. Regarding the first of these, I showed that over a wide range of c values, one can achieve 5% relative standard error (RSE) in K by obtaining enough heat to give a value of ~700 for the ratio of total heat to measurement precision, q_{tot}/σ_q. For $c > 1$, ΔH° is estimated with better precision than K, and for smaller c, the product $n \times \Delta H^\circ$ is similarly precise. To achieve adequate conversion of M to MX, I earlier obtained the empirical expression [1],

$$R_m = \frac{6.4}{c^{0.2}} + \frac{13}{c}, \text{but at least } 1.1, \quad (1)$$

for setting the ratio $[X]_0/[M]_0$ of total X to total M in the cell after the final (mth) injection.

5% precision is a reasonable practical goal in most ITC work. While much better precision is achievable, in principle, from instrumentation available today, in practice much routine work may have reliability less than 5% from systematic errors. For example, in an interlaboratory comparison study of an enzyme-inhibitor process, 14 experienced laboratories obtained results showing overall standard deviations (SDs) ten times larger than expected from the individual precision estimates [4]. Chodera and I showed that most of this excess dispersion could be explained by concentration errors of order 10% in the preparation of the reagents by the participating laboratories, with additional contributions from baseline errors likely in some cases [5].

Besides heat and product conversion, there is also the question of number of injections. I have shown that the notion, "more points is better" for data analysis, is wrong for ITC in the low-heat limit [1,6,7]. This is because a fixed amount of heat is being subdivided through the m injections, making the relative error in each q value go up as m while the statistical gain goes up only as $m^{1/2}$ [3]. For high-heat processes, this can reverse, because the nature of the data error changes for large q [6–8]; but then the predicted precisions are often unrealistically good when systematic effects are acknowledged. Accordingly, I have recommended the use of 10 injections in most work. This result enhances productivity in studies where many similar reactions are to be run, since 10-injection experiments can be completed much faster than 30-injection ones. Still, in high-heat situations (as in Fig. 1) it may be necessary to use more than 10 injections to stay within the instrument's range limitations. Similar considerations apply to very low-c processes, where variable volumes — small initially, increasing with m — better distribute the heat and yield better parameter precision [3,9].

To plan an ITC experiment, one must have some idea of likely values for K and ΔH°, *e.g.*, from other work on similar processes. Consider a reaction having dissociation constant $K_d = 10$ nM, giving $K = 10^8\ M^{-1}$. This is at the upper limit of ITC's capabilities, so conversion to product will not be an issue. If we use a relatively small $[M]_0 = 1$ µM, we have $c = 100$. Suppose $\Delta H^\circ = 10$ kcal/mol. Then $h\ (\equiv \Delta H^\circ \times [M]_0) = 0.01$ cal/l, and for the widely used MicroCal VP-ITC instrument (from Malvern Instruments since 2014), where the active cell volume is 1.4 ml, $q_{tot} = h \times V_0 = 14$ µcal. Using $\sigma_q = 0.2$ µcal [3], we obtain $q_{tot}/\sigma_q = 70$, and we can anticipate only crude estimates of the binding parameters from this experiment. On the other hand, if we can up $[M]_0$ to 10 µM ($c = 1000$), we will increase q_{tot}/σ_q to 700, which is our target for 5% estimation. However, for c this large, somewhat larger ratios are required for this precision, so we may need to settle for ~10% precision. At the other extreme, suppose we have $K = 100\ M^{-1}$. If we can set $[M]_0 = 1$ mM, we have $c = 0.1$, and Eq. (1) calls for a final concentration ratio of 140, which means a titrant concentration of $\sim 5 \times 140 \times 0.001\ M = 0.7$ M, since the total syringe volume is about a factor of 5 smaller than the cell volume. Even a modest ΔH° will indicate adequate heat in this case, but syringe concentrations this high are impossible in many biological processes, from solubility and other practical considerations. So we need to ask how well we can do with less complete titration, say, with 100 mM titrant concentration.

To facilitate such estimation, I provided in Ref. [3] a program that prompts the user for the kind of information used in these two examples and produces sample results with estimates of parameter precisions. This program utilizes the method of least squares (LS) in error propagation mode [10] — exactly fitting data with known uncertainty. For linear LS on data with Gaussian random error, the parameter standard errors (SE) are exact, meaning that, *e.g.*, Monte Carlo simulations will yield normally distributed parameter estimates having SD within statistical error of these predictions [11]. This result holds also for nonlinear LS (NLS), but only in the limit of small data error. With increasing data error, the parameter distributions deviate increasingly from Gaussian, and these deviations mean that confidence limits may not be simply obtained from the stated SEs. As is discussed below, in ITC this concern is significant for estimates of K at very high c, where its reciprocal K_d is the nearly normal parameter, and for ΔH° at low c, where the high correlation between n and ΔH° makes the product $n \times \Delta H^\circ$ well determined and $\Delta H^{\circ -1}$ approximately normal [1].

In subsequent sections, I review the basis for using LS in error propagation mode, also called experiment design; and I discuss how Monte Carlo simulations support such results for ITC. I then refine and generalize the earlier guidelines, providing a result that permits reliable estimation of the precisions for determining K, ΔH°, and n in all low-heat 1:1 binding situations having c in the range $10^{-3} - 10^4$. I briefly discuss the issue of dilution blanks and show how the simplest blank — water into water — can be used to estimate the data error σ_q needed for the ratio q_{tot}/σ_q that determines the parameter precisions. Finally, I illustrate the program provided for experiment design in [3] on examples from the recent literature. The program itself has been modified to include provision for a fourth parameter in the form of a constant background and is available in this new version in the supplementary material.

Since the present work follows directly from my 2012 paper [3], with a focus on just the most important results discussed there for 1–1 binding, I do not consider here the many earlier contributions to the topic of ITC experiment design, unless their results are specifically