Antibody characterization by isothermal titration calorimetry

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Isothermal titration calorimetry is a useful tool for the characterization of antibodies and antibody-related products, which uses heat as a detection medium, circumventing the problems associated with other analytical methods.

PHYSICAL characterization of biological products represents an important issue in research, as well as pharmaceutical and biotechnology product development. In particular, antibodies and their products are sometimes characterized by the strength or weakness of their affinity to specific antigens. Techniques that provide measures of affinity constants include isothermal titration calorimetry (ITC)[1], analytical ultracentrifugation[2], fluorometry[3], enzyme-linked immunosorbent assays (ELISA) and radioimmunoassays (RIA)[4], plasmon resonance methods (for example, BIAcore[5]), and equilibrium dialysis[6]. However, of those true 'in-solution' methods that do not require modification of the antibody or antigen (equilibrium, analytical ultracentrifugation and ITC), only ITC can provide the user with a complete thermodynamic characterization of the system under study. ITC measurements typically can be accomplished in approximately 30–60 min, are non-destructive to the sample, and can be applied over a broad range of solution conditions. This article will review the information that can be derived from ITC and detail the role ITC plays in the biophysical characterization of antibodies and antibody products.

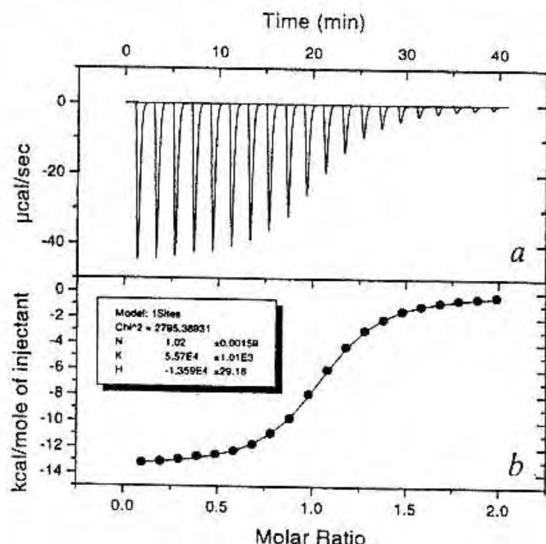

FIG. 2 Representative data from an ITC experiment. Panel *a* shows the raw heat data obtained over a series of injections. Panel *b* shows a binding isotherm created by plotting the areas under the peaks in panel *a* against the molar ratio of ligand added to macromolecule present in the cell. The box represents the fitted values for the stoichiometry, equilibrium constant, and enthalpy, respectively. The χ^2 statistic of the fit is given. The solid line represents a nonlinear fit using a 'one-set-of-sites' model.

The ITC experiment

ITC uses heat as a signal source. A syringe containing a solution of one element, commonly referred to as the 'ligand', is incrementally titrated into a cell containing a solution of the second element, commonly referred to as the 'macromolecule'. This is illustrated in Fig. 1, which contains a schematic of the ITC cell assembly. As the ligand is added to the macromolecule, heat is released or absorbed upon interaction of the two entities. The heat for each injection is measured by the ITC instrument and is plotted as a function of time over the injection series (Fig. 2*a*). As the macromolecule in the cell becomes saturated with ligand, the heat signal diminishes until at full saturation, where only background heat from the dilution of the ligand is observed.

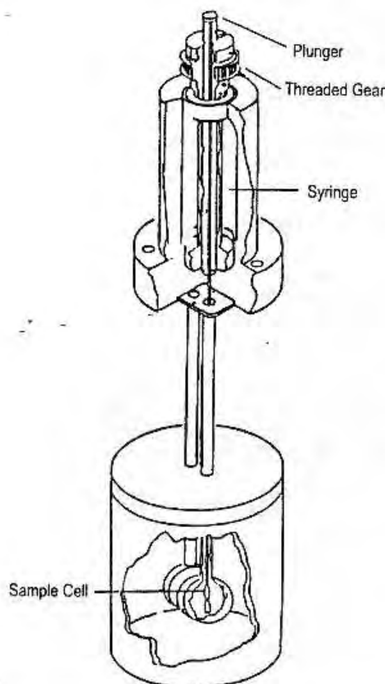

FIG. 1 Diagram of the ITC cell. A modified syringe containing the ligand rotates in place during the ITC experiment. The end of the syringe has been modified to provide continuous mixing. The plunger is computer-controlled and repeatedly injects precise volumes of ligand into the cell containing the macromolecule.

In a typical analysis, the total heat signal from each injection is determined by the area underneath the injection peak. When this total heat is plotted against the molar ratio of ligand added to macromolecule in the cell, as demonstrated in Fig. 2*b*, one obtains a complete binding isotherm for the interaction of ligand and macromolecule. With instruments such as the Microcal MCS-ITC system, the entire ITC experiment, from the timing and control of the injections to the acquisition of data, is entirely under computer control. The user inputs the experimental parameters (temperature, number of injections, injection volumes) and the computer carries out the experiment. After acquisition, the system can analyse the raw ITC data by application of the appropriate binding model. For a complete overview of the analysis and interpretation of ITC results see the reviews by Bundle and Sigurskjold[7] and Fisher and Singh[8].

Unlike other methods, ITC provides the user with a great deal of physical information about the binding process, typically from a single experiment. This information includes the binding affinity or binding equilibrium constant (K_{eq}), the molecular ratio or binding stoichiometry (n), and the heat or enthalpy (ΔH), as well as entropy (ΔS) of binding. The enthalpy of binding, stoichiometry, and free energy change (ΔG) of the binding process are determined by a nonlinear fit of the binding isotherm (Fig. 2*b*). The entropy of binding is determined from a difference between

the free-energy change and the enthalpy of binding. For more details see the article by Wiseman *et al.*[9].

Use in antibody engineering

Merely measuring the affinity between an antibody and an antigen leaves a great deal of useful and important information unexplored. A full thermodynamic analysis, when possible, provides a richer source of information, which can be used to interpret the molecular forces at work between the antibody and antigen. In one recent example, scientists at Genentech used the instrument described here to carry out thermodynamic investigations of the interaction of a large set of chimaerized anti-HER2 (heregulin) antibody Fab fragments with the $p185^{HER2\text{-}ECD}$ extracellular domain (ECD)[9–11]. All fragments were single amino-acid variants of the wild-type p185 ECD. The scientists demonstrated that, whereas in some cases affinity constants of the various fragments, determined by RIA or surface plasmon resonance, were essentially identical, their thermodynamic binding parameters (ΔH and ΔS) were very different. These differences provided substantial information regarding the specific molecular interactions occurring between the anti-HER2 Fab fragments and the p185 ECD, and formed the basis of further attempts to build stronger and more potent antibodies for use in cancer therapeutics. Perhaps most importantly, these scientists demonstrated that affinity constants alone could not provide enough information to engineer antibody fragments with full wild-type characteristics.

Characterization and detection

In antibody manufacturing processes, it is critical to identify lot-to-lot variations and variance outside acceptable limits. Typically, antibodies are characterized by measuring their titre alone. Less often, characterization is coupled with the determination of binding constants. It is hoped that titre data provide some indication as to how the antibody product will perform at the end of the manufacturing process. In contrast, ITC provides a full and accurate analysis of the binding interaction. One of the strengths of ITC is that measurements can be done under the same conditions and in the same state in which the antibody will ultimately be used. This is not true in other analytical techniques, where either the antibody or the antigen must be immobilized to a surface before measuring affinity[5]. Another advantage of ITC is its ability to measure affinities in heterogeneous media. It is not necessary to have a fully purified solution in order to detect binding. As long as background affinity is much lower than the affinity one is trying to measure, specific binding against a nonspecific background can be seen.

Heat is accurately described as a 'universal detector' of biological materials and binding phenomena. Unlike optical measurements in which only certain systems may absorb or emit at particular wavelengths, the generation or uptake of heat is a universal signature of all biological processes. Signals obtained by heat detection are comparable in sensitivity to those generated using optical detection. For example, concentrations of biological materials used in ITC experiments are similar to those used in absorbance-based techniques (ultraviolet/visible, analytical ultracentrifugation). In fluorometry, modification of one or both binding partners with a fluorescent tag is typically necessary for detection, sometimes altering the interaction one is trying to investigate. (Note that chemical tagging is physically analogous to surface coupling, which is required for surface plasmon resonance studies.) In RIA, putatively dangerous radioactive compounds are utilized as a signal source. ITC suffers from neither of these limitations.

Modern ITC technology continues to advance. Future instruments will have higher sensitivity and will require less sample. As has been demonstrated here, ITC is a valuable tool in the physical characterization of antibody products and plays an important role in antibody research laboratories and the antibody manufacturing processes. □

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1. Freire, E., Mayorga, O. L. & Straume, M. *Anal. Chem.* **62**, 950A–959A (1990).
2. Hensley, P. *Structure* **4**, 367–373 (1996).
3. Lakowicz, J. R. in *Principles of Fluorescence Spectroscopy* (Plenum, New York, 1983).
4. Goldberg, M. E. & Djavadi-Ohaniance, L. *Curr. Opin. Immunol.* **2**, 278–281 (1993).
5. Schuck, P. & Minton, A. *Trends Biochem. Sci.* **21**, 458–460 (1996).
6. Phizicky, E. M. & Fields, S. *Microbiol. Rev.* **59**, 94–123 (1995).
7. Bundle, D. R. & Sigurskjold, B. W. *Meth. Enzymol.* **247**, 288–305 (1994).
8. Fisher, H. F. & Singh, N. *Meth. Enzymol.* **259**, 194–221 (1995).
9. Wiseman, T., Williston, S., Brandts, J. F. & Lin, L.-N. *Anal. Biochem.* **179**, 5434–5441 (1993).
10. Kelley, R. F. *et al. Biochemistry* **31**, 5434–5441 (1992).
11. Kelley, R. F. & O'Connell, M. P. *Biochemistry* **32**, 6828–6835 (1993).