FUNDAMENTALS OF ISOTHERMAL TITRATION CALORIMETRY AND SOME APPLICATIONS

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Abstract. Very high affinity and very low affinity processes cannot be studied by standard protocols. Binding involving very small heat changes cannot be detected and sometimes, the large amounts of material required for accurate measurements make standard protocols impractical. Isothermal titration calorimetry (ITC) has become a gold standard in determining the binding energies and the data provided by ITC can be used as reference data by simulation and prediction methods of the thermodynamic variables. In this paper we will discuss the fundamental process of calorimetry, including a short history of isothermal titration calorimetry, a short description of the method and some applications of this technique.

Keywords: calorimetry, interactions, binding affinity, isothermal titration calorimetry

Isothermal Titration Calorimetry (ITC) is a thermodynamic technique used as an analytical tool to determine the binding affinity that occurs between two biomolecules as well as the thermodynamics of biological processes. ITC is largely considered as the gold standard assay for binding.

From historical point of view, the calorimetry has provided the first information on the characteristic of the energy transfer through heat. A large part of the empirical results obtained by calorimetry was the basis for formulating the thermodynamic principles and axioms, and other results formed the basis of calorimetric principles.

The starting point in measurement of the heat exchange was given by the experiment of James Joule who showed that the heat can be used as a form of energy, followed by chemist Joseph Black in the direction of heat exchange measurement. [Wigmans, 2003] The credit for creating the first modern techniques of calorimetry is given to the chemist Pierre Eugène Berthelot, who invented the term of endothermic and exothermic to describe the reactions of heat exchange. [Izatt et al., 1995] One of the pioneers in developing the art of calorimetry was W. Orthmann, [Hatta, 1997] who patented a differential calorimeter that could determine heat transfers of the order of μWatt.

Over the past 15 - 20 years, the sensitivity of these instruments has improved dramatically [Kraftmakher, 2004]

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Figure 1. A schematic representation of Isothermal Titration Calorimeter
An isothermal titration calorimeter (Figure 1) is composed of two identical cells (sample cell and reference cell) made of a highly efficient thermal conducting material such as Hastelloy, shielded by an adiabatic jacket, a syringe fitted with a stirrer, an electrical motor which continuously rotates the entire injection-agitation system with a preselected speed to ensure the homogeneity of the mixture from measurement and a sensitive thermopile/thermocouple circuit who detect the temperature difference between the two cells and between the cells and the jacket [ROSELLIN et al. 2010]. In modern ITC instruments, the cell volumes are nominally 1.5 mL, the temperature of the thermostat can be set from about 5 to 80 °C, the injected volume can range from about 1 to 20 μL, and heats as small as 0.4 μJ (0.1 μcal) can be measured [BJELIC and JELESAROV, 2008; LEWIS and MURPHY, 2005; PIERCE et al., 1999].

A typical ITC experiment is performed at constant temperature as follows: a binding solution (“ligand” or titrant, such as a drug, protein, DNA molecule,) [FEIG, 2007; PIERCE et al., 1999] is titrated into cell measurement containing a solution of the macromolecule. After each injection of the ligand into the cell of the calorimeter, the two solutions are interacting, and the heat is released or absorbed in direct proportion to the amount of binding. The experimental data can be plotted as the total heat accumulated up to injection $i$ normalized to the total ligand concentration at step $i$ against the ratio of the total ligand concentration at step $i$ to the total receptor concentration. [PERMYAKOV AND KRETSINGER, 2011; CAMPOY et al., 2004]

Each separate peak reflects the amount of heat evolved with the injection of a small aliquot of ligand into the ITC cell. As the ligand is titrated into the ITC cell, the amount of heat released or absorbed is proportional to the amount of binding. When the system will come to saturation, the heat signal will diminish, until only heats of dilution are observed.

![Figure 2. Schematic representation of thermogram and titration isotherm](image)

The shape of the titration curve offers useful information on the strength of the binding interactions and the number of ligands recognized by the macromolecule.
The ITC signal is dependent on the concentration of the macromolecule, $[M]$, and the ligand $[L]$, the cell volume, the injected volume, and the values of $K$, $\Delta H$, and $n$. [CHAVELAS et al. 2006; LEWIS and MURPHY, 2005] Low concentration values lead to a isotherm almost horizontal, indicating a weak binding of ligand to macromolecule site, without allowing to obtain a conclusive value of enthalpy and the affinity constant; high concentration values lead to a isotherm almost rectangular, entire amount of ligand will be linked to the macromolecule, allowing determination of enthalpy which is characterized by a great value, but doesn’t allow the determination of affinity constant [LEWIS and MURPHY, 2005; PIERCE et al., 1999].

The advantages of using ITC are rising from its simple but precise technique:

• ITC is an easy to use instrument. After loading the sample, it may run unattended. The technician involvement is minimized and the operating software provides accurate information.
• ITC experiments provide a complete view of all of the binding parameters in a single experiment: number of binding sites ($n$), binding affinity $K_d$ in range of milimolar to nanomolar, enthalpy change ($\Delta H$), and entropy ($\Delta S$) of binding;
• ITC experiments investigate any biomolecular interaction, the calorimeter being an universal detector, as almost all chemical reactions take place with a change in heat;
• Directly measurement of sub-millimolar to nanomolar binding constants ($10^2$ to $10^9$ M$^{-1}$). Measurement of nanomolar to picomolar binding constants ($10^8$ to $10^{12}$ M$^{-1}$) using the competitive binding technique;

Nowadays ITC is used to characterize of molecular interactions of small molecules, protein [9], antibodies, nucleic acids, lipids and other biomolecules (protein-small molecule, protein – protein [PIERC et al., 1999], target - drug, enzyme - inhibitor, antibody - antigen, protein - DNA / RNA [BHELIC and JELUSARON, 2008; FEIG, 2007; SALDM and FEIG, 2008]), protein - lipid, small molecule - small molecule). [CAMPOY et al. 2004]. Also, some of the applications of isothermal titration calorimetry are: lead optimization, enzyme kinetics, assessment of the effect of molecular structure changes on binding mechanisms and assessment of biological activity. The information provided by ITC experiments is of great help in the development of pharmaceutical compounds.

Over the last two decades isothermal titration calorimetry has become increasingly popular for study of all kind of binding reactions including protein - ligand interactions and peptide - membrane interactions [LIANG, 2008]. Isothermal titration calorimetry allows measurement of the binding and equally gives a complex thermodynamic analysis of the binding reaction, including the free energy of binding $\Delta G$, the enthalpy of binding $\Delta H$, the entropy of binding $\Delta S$ and the heat capacity change $\Delta C_p$ [WIEPRECHT, 2003]. Isothermal titration calorimetry has proven to be extremely sensitive in investigation of secondary processes that are correlated with protein - membrane binding, such as detecting changes and differences in bilayer structure, differences between curved an planar membranes, membrane permeabilization, peptide - induced lipid phase transition, peptide aggregation at the membrane surface and peptide conformational changes [BJELIC et al. 2008; WIEPRECHT, 2003].

Another useful application of this technique is the utilization in the study of the interaction between the therapeutic agents (drugs) and the macromolecules, such as the proteins found in blood. One of the biggest advantages of isothermal titration calorimetry is the fact that it can precisely measure the thermodynamic and kinetic parameters of the interaction between the drug molecules and the macromolecules, as well as the binding site location. Once the drugs are being introduced in the circulation system, they can bind to diverse blood components, such as the blood proteins. This binding has an important influence on the pharmacodynamics and the toxicological action of drugs and on the volume of distribution of a drug [Caldwell and Yan, 2005; WEBER et al., 2003]. Generally, at the beginning of the experiment the adiabatic shell temperature is set at a constant temperature. Then, in the measurement cell containing the protein solution having a given concentration is titrated an exact amount of
drug. The two components of interest interact, leading to binding of most of the drug molecules to the protein molecules. This first injection generates a large amount of heat while for the following injections there are increasingly fewer protein molecules available for binding and therefore less heat of binding is generated. After several injections almost all of the protein binding sites are occupied by drug molecules, and the system is reaching saturation.

Furthermore, ITC is equally used in pharmacological research for the study of specific interactions such as the antigen-antibody or enzyme-substrate. [BJELICA and JELESAROV, 2008]. With the ITC can quickly and efficiently be determined various parameters such as antibody affinity (Kₐ), heat released from antigen binding to antibody (ΔH), the number of active binding sites (n) of the antibody. The structural changes could be linked to the changes in binding affinity. Moreover, the heat generated from binding can be a useful instrument in the study and prediction of the reaction of antigen - antibody in vitro and in vivo.

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