Recent trends and some applications of isothermal titration calorimetry in biotechnology

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Review

Recent trends and some applications of isothermal titration calorimetry in biotechnology

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Isothermal titration calorimeters (ITCs) are thermodynamic instruments used for the determination of enthalpy changes in any physical/chemical reaction. This can be applied in various fields of biotechnology. This review explains ITC applications, especially in bioseparation, drug development and cell metabolism. In liquid chromatography, the separation/purification of specific proteins or polypeptides in a mixture is usually achieved by varying the adsorption affinities of the different proteins/polypeptides for the adsorbent under different mobile-phase conditions and temperatures. Using ITC analysis, the binding mechanism of proteins with adsorbent solid material is derived by elucidating enthalpy and entropy changes, which offer valuable guidelines for designing experimental conditions in chromatographic separation. The binding affinity of a drug with its target is studied by deriving binding enthalpy and binding entropy. To improve the binding affinity, suitable lead compounds for a drug can be identified and their affinity tested by ITC. Recently ITC has also been used in studying cell metabolism. The heat produced by animal cells in culture can be used as a primary indicator of the kinetics of cell metabolism, which provides key information for drug bioactivity and operation parameters for process cell culture.

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1 Introduction

All physical, chemical and biological processes are accompanied by heat exchange. The enthalpy change of a reaction, ΔH°, is a fundamental thermodynamic quantity that describes the amount of heat released or absorbed during the course of the reaction. The ΔH° of a reaction can, in general, be determined in one of the two ways; it can be determined directly using calorimetry or indirectly by van't Hoff method. For a reversible reaction the van't Hoff relationship is described by Eq. (1),

$$\Delta H^0 = -R\left(\frac{\partial \ln K}{\partial 1/T}\right) \quad (1)$$

where R is the gas constant, T is the absolute temperature (at constant pressure). The instantaneous slope of a plot of lnK *vs*. 1/T, multiplied by -R, is used to determine ΔH° [1].

Differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC) are the only methods for the direct determination of ΔH. DSC measures the heat capacity of a reaction as a function of temperature. The heat capacity of the system is then measured against temperature as the direct thermodynamic observable. Therefore, in a single DSC experiment, transition midpoint – Tm, enthalpy (ΔH) and heat capacity change (ΔCp) can be determined. ITC is the direct method to measure

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Abbreviations: **EDL**, electrical double layer; **HEWL**, hen egg white lysozyme; **HIC**, hydrophobic interaction chromatography; **IEC**, ion-exchange chromatography; **IMAC**, immobilized metal ion affinity chromatography; **ITC**, isothermal titration calorimetry/calorimeter

the heat change in a reaction at constant temperature. In ITC analysis, the measurement of heat is made at a given temperature, which allows direct determination of binding constants (K_a), reaction stoichiometry (n), enthalpy change (ΔH), free energy change (ΔG) and entropy change (ΔS). In addition, varying the temperature of the experiment allows the determination of the heat capacity (ΔC_p) for the reaction, thereby providing a complete thermodynamic profile of the molecular interaction.

When the van't Hoff enthalpy values for a particular reaction are compared to enthalpy for the same reaction obtained directly by calorimetric methods, there is often a significant difference between the two values. Furthermore, the binding mechanism may differ with temperature, and the heat capacity of the molecules involved may also vary with operating temperature. For example, the binding enthalpy of cytidine 2′-monophosphate (2′-CMP) to ribonuclease A (RNase A) revealed that the enthalpy obtained from the van't Hoff equitation (ΔH_{vH}) differed from those obtained from microcalorimetric measurement (ΔH_{ITC}) at various temperatures and binding amounts [2]. This indicates that the binding reaction is more complex than the simple one-to-one binding model. The direct measurement of ΔH_{ITC} represents the total energy change in the whole adsorption process, which includes dilution heat, binding heat, the energy required for desolvation, and the energy change after protein rearrangement. In contrast, ΔH_{vH} is calculated on the basis of the reversible equilibrium process, and the analysis does not consider the contribution of protein conformation change. Furthermore, the ΔH_{vH} is calculated from the free energy change at different temperatures. Since protein conformation is sensitive to temperature, ΔC_p varies with temperature, causing variation of ΔH_{vH}. Therefore, major contributing factors to the discrepancy between the ΔH_{vH} and ΔH_{ITC} are difference in the mechanism of protein adsorption caused by the changes in protein conformation and the interaction with solvent molecules and the hydrophobic ligand [3]. Weber has discussed the reasons for the differences between van't Hoff and calorimetric enthalpy values in detail [4]. Chaires [5] and Horn *et al*. [6] have noted that the enthalpy observed by van't Hoff analysis is considerably lower than that for calorimetric enthalpy and this discrepancy is associated with the reduced precision of van't Hoff analysis. Therefore, ITC is considered an accurate method for estimating the total heat change in various biological reactions. ITC is mostly used to measure strengths of interaction between proteins and ligands: either small molecules like drugs, or large ones such as nucleic acids or other proteins. The biotechnological issues to which microcalorimetry has been successfully applied includes antibody studies [7]; cell metabolism [8]; drug receptor interactions [9]; enzyme interactions, *e.g*., with co-enzymes [10], inhibitors [11], substrates [12], and drugs [13]; antigen-antibody interactions [14]; DNA-drug interactions [15]; protein interactions, *e.g*., with other proteins, lipids, carbohydrates, DNA, ligands, and nucleic acids [16–20]; and biopolymer interactions, *e.g*., with other biopolymers, drugs, ligands, [21–23], and enzyme activity analysis [24].

This review article reports our experiences and other reported results on ITC in the investigation of binding interaction between protein and solid surfaces for its application to bioseparation by liquid chromatography. In addition, we give a brief discussion on the application of this technique in drug development and studying cell metabolism.

2 Instrumental methodology

The calorimetric method for determination of binding constants was first developed in the early 1960s for the evaluation of thermodynamics of proton ionization from weak acids and bases, and for metal-ligand binding reactions [25]. Design improvements were made in an adiabatic calorimeter with no heat exchange taking place between the calorimetric vessel and its surroundings. The amount of heat that is evolved or absorbed in an ideal adiabatic calorimeter is equal to the product of the measured temperature change and the heat capacity of the vessel, including its content. A wide range of calorimeters are available to meet different needs of the user [26–29]. A typical isothermal titration calorimeter is composed of two identical cells (a reference and a sample cell) made of highly efficient thermal conducting material surrounded by an adiabatic jacket. Sensitive thermopile/thermocouple circuits are used to detect temperature differences between the reference cell (filled with buffer or water) and the sample cell containing the macromolecule. Known concentrations of degassed samples are taken in the injector and sample cell. Prior to the injection of the titrant, a constant power (<1 mW) is applied to the reference cell. This signal directs the feedback circuit to activate the heater located on the sample cell. This represents the baseline signal. During the experiment, the titrant is added into the sample cell in precisely known aliquots, causing heat to be either taken up or evolved. For an exothermic reaction, the temperature in the sample cell will increase, and the feedback power will be deactivated to maintain

equal temperatures between the two cells. For endothermic reactions, the reverse will occur, *i.e.*, the feedback circuit will increase power to the sample cell to maintain the temperature.

ITC instruments should be routinely calibrated by applying specified electrical pulses of approximately 5–10 cal/s. The total heat as determined by the area under the pulse should be within 2% of the expected value (Omega ITC Manual, Microcal Inc.). Baranauskienė *et al*. [30] have reported several chemical reactions to validate ITC equipment. Joel Tellinghuisen [31] used the heat of dilution of NaCl (aq) to calibrate a Micro Cal model (VP-ITC) instrument. The performance of the calorimeter can also be tested by measuring the heat of a standard chemical reaction, such as the protonation of tris(hydroxymethyl)aminomethane (THAM) by HCl [32]. It is advisable to use both instrumental calibration and one chemical calibration method to calibrate the instrument.

Measurements consist of the time-dependent input of power required to maintain equal temperature between the sample and reference cells. Observations are plotted as the power needed to maintain the reference and the sample cell at an identical temperature. This power is given as a function of time in seconds. The raw data for an experiment consists of a series of heat flow peaks (power), with every peak corresponding to a ligand injection. These heat flow peaks are integrated with respect to time, giving the total heat effect per injection. Peak integration is made either in a manual peak-by-peak fashion or automatically using the ITC software package. Baseline selection is an important factor in ITC data analysis and user input in the automated integration routine is limited. Therefore, manual peak-by-peak integration in which the operator defines the baseline regions used in the integration is preferred. This heat released or absorbed is in direct proportion to the amount of binding that occurs. Additionally, a volume correction is also performed due to dilution in the sample cell due to each injection.

Interpretation of calorimetric data always involves a model, usually expressed as an equation or set of equations. In the case of a receptor with multiple binding sites of different affinity, statistical thermodynamic treatment of the data is required. The amount of heat evolved on addition of ligand can be represented by Eq. (2) [33]:

$$Q = V_0 \Delta H_b [M]_i K_a [L] / (l + K_a [L]) \quad (2)$$

where V_0 is the volume of the cell, ΔH_b is the enthalpy of binding per mole of ligand, $[M]_t$ is the total macromolecule concentration including bound and free fractions, K_a is the binding constant, and [L] is the free ligand concentration. For a more general model of binding, the multiple independent sites model, the macromolecule contains multiple ligand binding sites that are noninteracting. The total heat of binding can be described by

$$Q = V_0 [M]_i \Sigma (n_i \Delta H_i K_{ai} [L]) / (l + K_{ai} [L]) \quad (3)$$

A detail discussion of the derivation of the above equations is presented in [33]. The method of analysis will be specific to the system under investigation and it is advised to attempt fitting according to a general model before using more specific models. The pattern of these heat effects as a function of the molar ratio [ligand]/[macromolecule] can then be derived to give the thermodynamic parameters.

The thermodynamic parameters are derived as follows:

$$\Delta G = \Delta G^0 + RT \ln Ka \quad (4)$$

At equilibrium $\Delta G = 0$

$$\therefore \Delta G^0 = -RT \ln Ka = \Delta H^0 - T\Delta S^0 \quad (5)$$

where ΔG, ΔG^0, ΔH^0, ΔS^0 and K_a are free energy change, standard free energy change, standard enthalpy change, standard entropy change and affinity constant, respectively. The affinity constant K_a is derived from isotherm study. As ΔH is dependent on concentration and is not considered as standard state (at standard state [conc] = 1 M), the ΔH value is directly measured by ITC analysis.

From ΔG^0 and ΔH, ΔS^* is derived as follows [34].

At extreme dilution condition

$$\Delta S^* = \Delta S_{unitary} + \Delta S_{cratic} \quad (6)$$

$\Delta S_{unitary}$ is given by Eq. (7) [35]

$$\Delta S_{unitary} = \Delta S^0 - \Delta S_{cratic} = \Delta S^0 - R \ln X_M \quad (7)$$

Where, $\Delta S_{unitary}$ is independent on concentration. $\Delta S_{cractic}$ is dependent on concentration. $\Delta S_{cractic}$ is given by Eq. (8)

$$\Delta S_{cratic} = R \ln X_M \quad (8)$$

X_M is the concentration of water in dilute aqueous solution, which is given by a value 55.6 M [36] and so the $\Delta S_{cractic}$ is calculated as –7.98 cal $mol^{-1}K^{-1}$. From this, $\Delta S_{cractic}$ was derived and $\Delta G_{unitary}$ was obtained from Eq. (9).

$$\Delta G_{unitary} = \Delta H + T\Delta S_{unitary} \quad (9)$$

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3 Examples of ITC applications in biotechnology

3.1 ITC applications in bioseparation

The chromatographic separation process is based on the difference in the surface interactions of the analyte and eluent molecules. There are two basic approaches for thermodynamic description of the HPLC retention phenomena, one is based on the partitioning theory and another is based on adsorption. Partition is a concentration change in the system due to the distribution of the components between two (or more) phases and adsorption is the concentration change in the system in presence of interface with another phase and due to the surface forces. One of the main problems with chromatographic separation is the difficulty in the optimization of different parameters to design effective process for protein separation/purification, which is presently fixed by trial and error. Knowledge of the binding mechanism of proteins at the liquid-solid interface provides valuable insights into the separation and purification of proteins. The separation/purification of specific proteins or polypeptides in a mixture is usually achieved by varying the adsorption affinities of the different proteins/polypeptides for the adsorbent. The thermodynamic characteristics provide valuable information for understanding the binding mechanism [37].

The feasibility of a protein or polypeptide being adsorbed (at a constant pressure P, and temperature T) is determined by the change in the standard Gibbs free energy, ΔG^o, of the system. The relationship between standard enthalpy (ΔH^o) and entropy (ΔS^o) changes, the equilibrium binding affinity constant, (K_a) and the corresponding standard Gibbs free energy change can be expressed by Eq. (5). Proteins or polypeptides can be separated by varying the magnitudes of the respective enthalpy and/or entropy changes, resulting in different binding affinities and different free energy changes. Using microcalorimetry, the heat of protein adsorption onto solid surfaces can be measured. The heat measured can be further quantified by the amount of protein adsorbed, which is obtained from the equilibrium binding isotherms. The heat of protein adsorption onto solid surfaces can be measured by ITC [38–50]. In this experimental technique, the adsorbent is suspended in the equilibrium buffer solution and placed in the sample cell: it is then titrated by protein solution prepared in the equilibrium buffer solution. The output signals are collected as power (P) *vs*. time (t) and are integrated and quantified by the amount of bound protein to give the enthalpy change of adsorption. The adsorption enthalpy change can be calculated as:

$$Q_{ads} = V \times q^* \times \Delta H_{ads} \quad (10)$$

where Q_{ads} denotes the net heat attributed to the interaction between proteins and adsorbents (after corrected by the dilution heat of proteins and adsorbents) [J], V represents the volume of the adsorbent in the ampoule (mL), q denotes the amount of bound protein (mol/mL), and ΔH_{ads} is the enthalpy change (kJ/mol).

Using isothermal calorimetric technique, detailed thermodynamic characteristics have been reported on hydrophobic interaction chromatography (HIC), immobilized metal ion affinity chromatography (IMAC), and ion-exchange chromatography (IEC).

The binding mechanism can be explained by considering five sequential subprocesses: (a) exclusion of water molecules or ions surrounding the protein surface, *i.e.*, the dehydration or deionization (removal of the electrical double layer, EDL) subprocess of the protein; (b) exclusion of water molecules or ions surrounding the adsorbent, *i.e.*, the dehydration or deionization subprocess of the adsorbent; (c) the hydrophobic interactions between the protein and the hydrophobic HIC adsorbent (in HIC); coordination interaction and/or nonspecific interaction formed between protein and the immobilized metal ion (in IMAC); electrostatic interactions and/or nonspecific interactions, *e.g.*, hydrophobic interactions between the protein and the ion exchange adsorbent (in IEC); (d) the structural rearrangement of the protein upon adsorption/partition; and (e) the structural rearrangement of the excluded water molecules or ions in the bulk solvent.

3.1.1 Hydrophobic interaction chromatography

ITC analysis is helpful in choosing a suitable hydrophobic adsorbent, salt type and salt concentration for separation/purification of polypeptide or proteins by HIC [38–43]. In addition, the values of adsorption enthalpy (ΔH) for different proteins provide guidelines to separate these proteins by HIC. For example, ITC analysis revealed that adsorption enthalpies (ΔH) of α-chymotrypsinogen A with two adsorbents (CM-butyl and CM-octyl Sepharose) were lower than those of trypsinogen, in which α-chymotrypsinogen A had a larger exposed hydrophobic area than trypsinogen (Figs. 1 and 2) [39]. These can be result from a higher exothermic amount of hydrophobic interaction and reduction in the endothermic amount for structural rearrangement of α-chymotrypsinogen A than

for trypsinogen. As a result, α-chymotrypsinogen A has a longer retention time than trypsinogen and are separated by HIC. In another study, peptides consisting of glycine and different lengths of tryptophan such as, GWG, GWWG, GWWWG were investigated for its adsorption behavior with hydrophobic adsorbents such as, octyl Sepharose and CM-octyl Sepharose [43]. Tryptophan is an unusual amino acid and plays an important role in biological systems [51–53]. The binding mechanism of tryptophan adsorption on octyl Sepharose and CM-octyl Sepharose was elucidated by equilibrium binding analysis, dilution heat of peptide solution and adsorption enthalpy. The mechanisms of CM-octyl Sepharose adsorption with GWG and GWWG involve hydrophobic and electrostatic interactions. However, the binding of GWWWG with the adsorbent is dominated by the hydrophobic interaction. These findings suggest that tryptophan chain length or the number and hydrophobicity of amino acid residues of a peptide is critical for the peptide binding and solvation mechanism. The thermodynamic data for peptides containing different tryptophan chain length offer fundamental information for protein separation and peptide design application.

ITC analysis is helpful in choosing adsorbents and provides valuable guidelines to design experimental parameters for the specific adsorbent for separating the proteins or polypeptides. Hydrophobic interaction differs for adsorbents with different chain length, type and density. Alkyl-based and phenyl-based adsorbents differ in their adsorption affinity for proteins and this can be result in different interaction mechanisms. Compared to n-alkyl-based HIC adsorbents, the interaction between proteins and HIC adsorbents containing aromatic groups such as phenyl or benzyl ligands follow a less predictable binding behavior, attributed to the participation of π-π interactions [54]. Comparison of the equilibrium binding affinity for alkyl and phenyl adsorbents with hen egg white lysozyme (HEWL) showed that the adsorption affinity of the HEWL with the Toyopearl butyl adsorbents was larger than that found with the Toyopearl phenyl adsorbent [39]. ITC analysis illustrated that both adsorption enthalpy (ΔH_{ads}) and adsorption entropy (ΔS_{ads}) values for Toyopearl butyl adsorbent with HEWL were higher than that with the Toyopearl phenyl adsorbent. The endothermic amount of the heat associated with the dehydration and the reduction in the EDL (*i.e.*, the a and b subprocesses) was smaller when the Toyopearl butyl adsorbent was used in comparison to the Toyopearl phenyl adsorbent. The binding strength associated with the hydrophobic interactions of HEWL with the Toyopearl phenyl adsorbent was stronger than that with the Toyopearl butyl adsorbent, leading to an increase in the exothermic amount of heat (*i.e.*, the c subprocess). This result provides evidence to support the conclusion that chromatographic processes based on the hydrophobic interactions are mostly driven by the entropy gain. This suggests that under these conditions separation/purification of proteins or polypeptides can be performed by considering temperature as the key parameter.

In another study, n-alkyl-based HIC adsorbents, such as the butyl- and octyl-containing adsorbents were compared [39, 40, 42]. The microcalorimetric results confirmed that the adsorption of two proteins (α-chymotrypsinogen A and trypsinogen) on CM- butyl Sepharose was exothermic, while its adsorption on CM-octyl Sepharose was endothermic.

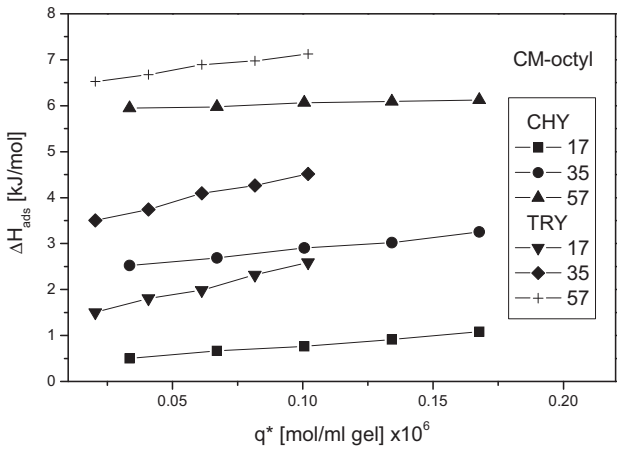

Figure 1. Adsorption enthalpies (ΔH) of α-chymotrypsinogen A (CHY) or trypsinogen (TRY) onto CM-butyl Sepharose at various ligand densities, 4.0 M NaCl, and pH 10.0 (the number in the key of the figure represents the ligand density of the adsorbent in mol/mL) [39].

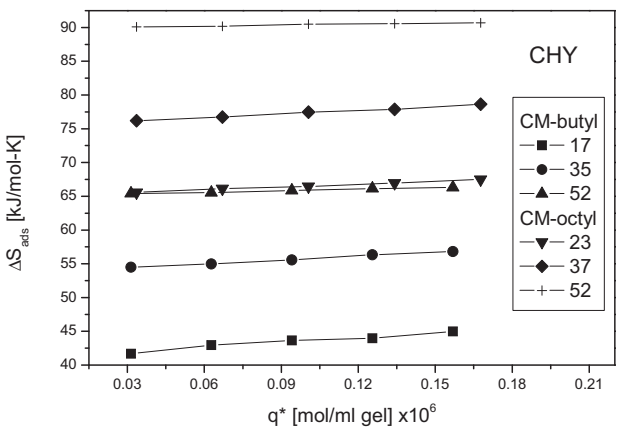

Figure 2. Adsorption enthalpies (ΔH) of α-chymotrypsinogen A (CHY) or trypsinogen (TRY) onto CM-octyl Sepharose at various ligand densities, 4.0 M NaCl, and pH 10.0 (the number in the key of the figure represents the ligand density of the adsorbent, in mol/mL) [39].

The adsorption per mole of these proteins onto CM-octyl Sepharose required more energy for dehydration than that with CM-butyl Sepharose, and higher entropy gain with CM-octyl Sepharose as compared with CM-butyl Sepharose. Therefore, the interaction mechanism of the protein with CM-octyl Sepharose is more like a partitioning event, while with the CM-butyl Sepharose the mechanism is an adsorption-dominated process. Therefore, when CM-octyl Sepharose is used as the adsorbent, temperature is a powerful parameter that can be adjusted for better separation/purification of proteins or polypeptides by HIC.

The adsorbents CM-octyl Sepharose and octyl Sepharose have similar ligand density and both have exposed OH groups on the surface. However, CM-octyl Sepharose has partially unreacted carboxyl groups that remain on the surface. The adsorption behavior and thermodynamic parameters with octyl Sepharose and CM-octyl Sepharose with the oligomers (GWG, GWWG, GWWWG) showed that the binding affinities of each peptide for the two adsorbents are similar. However, the mechanism of adsorption for peptides with these hydrophobic ligands is quite different with respect to the binding enthalpy between peptides and adsorbents [43]. The adsorption of the peptides on octyl Sepharose is an entropy-driven process for all the peptides and is governed by hydrophobic interaction. In contrast, with CM-octyl Sepharose hydrophobic and electrostatic interactions are both included in the binding operation for GWG and GWWG. However, the binding of GWWWG with the adsorbent is dominated by the hydrophobic interaction. This finding is useful for fixing the eluent gradient and temperature for HIC separation. When both hydrophobic and electrostatic forces influence the interaction between proteins and the adsorbent, two parameters, such as temperature and salt concentration, are taken into consideration and are adjusted based on the relative values of ΔH_{ads} and ΔS_{ads}. When hydrophobic force alone influences the interaction between proteins and the adsorbent, good separation/purification of protein/polypeptide can be achieved by selecting suitable temperature.

Temperature plays an important role in separation/purification of proteins by HIC [55]. At different temperatures the interaction may be dominated by partitioning, adsorption, or both. Partitioning implies that the protein molecule increases exposure of the hydrophobic residue of the inner protein core to aqueous solution and penetrates the solid surface, whereas adsorption implies that the protein molecule is simply in surface contact with the adsorbent. ITC analysis helps in determining the binding mechanism and in choosing a suitable temperature for separation/purification of proteins by HIC. For example, the affinity and enthalpy differences between two proteins (α-chymotrypsinogen A and trypsinogen) with two adsorbents (butyl Sepharose and octyl Sepharose) under varying temperatures showed that the adsorption quantity of the proteins with these Sepharose adsorbents increased when the temperature was increased from 298 to 310 K [38, 42]. But ΔH_{ads} value of α-chymotrypsinogen A with butyl Sepharose was increased, while the ΔH_{ads} value of trypsinogen was reduced. Comparison of the value of adsorption entropy (ΔS_{ads}) showed that the α-chymotrypsinogen A exhibited higher value than trypsinogen. The higher entropy gain was mainly from the dehydration process, which characterized the partitioning process. This observation implies that as the temperature is increased, the solution polarity is reduced, and the decreased stability of the protein structure and the increased exposure of the hydrophobic residue of the inner protein core to aqueous solution lead to interaction mechanism changes from an adsorption-dominated process to a partitioning process. In addition, for octyl Sepharose, the ΔH value of α-chymotrypsinogen A was positive, but decreased with temperature increment. However, the ΔH value of trypsinogen was positive and increased with temperature. Therefore, as the temperature increases, the interaction mechanism of the proteins for octyl Sepharose is a partitioning-dominated process. These observations are useful for understanding the temperature and eluent gradients required for chromatographic separation/purification of proteins/polypeptides.

The retention of protein is achieved by varying the salt concentration in the aqueous mobile phase. Horvath and co-workers [56] demonstrated that, in the absence of special binding effects, an increase in the salt molality in the mobile phase or the use of a salt with a greater molal surface tension increment will result in the increased retention. The molal surface tension increment is a concentration-independent characteristic of the salt that relates to its water-structuring potential. Thus, the molal surface tension increments of salts have been reported to highly correlate with their position in the lyotropic series [57], which is an empirical relationship discovered by Hofmeister [58] linking the solution properties of salts and their effect on the solubility of proteins. It has been noted that certain salts enter into specific interaction with proteins. The effect of salt type on protein retention can be related to the molal surface tension increment of the salt [56]. ITC analysis provides a clear picture

of the retention characteristics of different salt types. Higher affinities were obtained in the presence of Na_2SO_4 compared to $(NH_4)_2SO_4$ in two adsorbents (Toyopearl butyl and Toyopearl phenyl) [39]. ITC analysis demonstrated that lower values of ΔH_{ads} were obtained in the presence of Na_2SO_4 when compared to $(NH_4)_2SO_4$ due to the greater ability to decrease the endothermic component of the dehydration of the protein and HIC adsorbent and to reduce the EDL (*i.e.*, the a and b subprocesses). In addition, the exothermic amount of heat (*i.e.*, the c subprocess) with Na_2SO_4 was higher than with $(NH_4)_2SO_4$. This resulted in enhanced interaction between protein and the HIC adsorbent when Na_2SO_4 was used. Since Na_2SO_4 has a higher molal surface tension increment, a more solvophobic environment will occur for HEWL (and the other proteins) in an aqueous solution containing Na_2SO_4 rather than $(NH_4)_2SO_4$. Thus, salts with higher molal surface-tension increments produce increased retention at equal molal salt concentrations. The retention of proteins on hydrophobic columns can be controlled by adjustment of salt concentrations [59]. The adsorption affinity of the myoglobin with either butyl or octyl Sepharose was increased with the salt concentrations [40]. ITC analysis illustrated that the ΔH_{ads} of myoglobin adsorption onto butyl and octyl Sepharose were all decreased with increase in $(NH_4)_2SO_4$ concentration. This was due to the heat revealed by van der Waal's forces of the protein with the adsorbent (*i.e.*, the (c) process) increased with $(NH_4)_2SO_4$ concentrations. The increase of ΔH_{ads} value was compensated by the larger positive value of ΔS_{ads}. For a better separation/purification of protein/polypeptide by HIC, concentration of salt and temperature can be adjusted by considering the ΔH_{ads} and ΔS_{ads} values.

3.1.2 *Immobilized metal ion affinity chromatography*

ITC measurement can also find application in IMAC. The interaction mechanism of a protein with an immobilized metal ion is affected by the topography of the protein surface through various parameters, such as type of metal ion, pH of the medium and ionic salt concentration. ITC analysis is helpful for choosing a suitable metal ion and to set up suitable conditions for the chosen metal ion in IMAC [45-49]. The ΔH_{ads} values of lysozyme adsorption onto immobilized Fe(III) are higher than those of Cu(II) at various pH values (Fig. 3) [48]. The heat required for the dehydration of Cu(II) is lower than that for the dehydration of Fe(III), and the heat generated from the formation of the coordination with Cu(II) is higher than with Fe(III). The coordinate bond formation of nitrogen atoms with

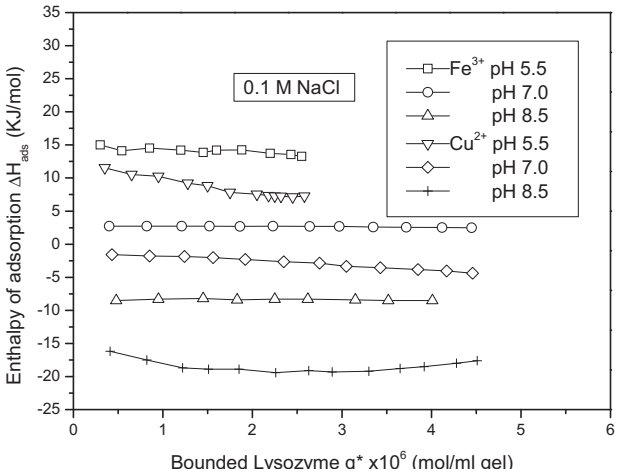

Figure 3. Enthalpy of adsorption (ΔHads) of lysozyme onto CS-IDA-Fe(III) or CS-IDA-Cu(II) at various pH values, 0.1 M NaCl, and 20 mM phosphate [48].

Cu(II) is more competent than with Fe(III), implying that the coordination of the nitrogen atom with Cu(II) is more thermodynamically stable than with Fe(III). The adsorption affinities and retention of proteins fused with histidine tags towards immobilized metal ions increased with the number of exposed histidines. The ΔH_{ads} values of SjGST/His adsorption onto the Ni-NTA were lower than that of SjGST [49], because SjGST/His has an additional fusion tag containing six adjacent histidines in comparison to SjGST, which coordinated with immobilized Ni^{2+} resulting in a reduction in ΔH_{ads} values. As a result, SjGST/His was retained more strongly than SjGST. This suggests that the retention of each protein can be adjusted on the basis of histidine tags.

3.1.3 *Ion-exchange chromatography*

In IEC, the binding strength of the protein with the ion-exchange resin can be affected by numerous factors, including pH value, salt concentration, and temperature, *etc.*, and these effects have been widely investigated [60, 61]. The thermodynamic data revealed that at a pH nearer to the p*I* value of protein, both electrostatic and hydrophobic forces mutually affect the interaction behavior in IEC [44, 50]. Similar observations are also made by Dieterle *et al.* [62] in adsorption studies of two human monoclonal antibodies on two different strong cation exchange resins.

3.2 ITC applications in drug development

ITC has an important application in the characterization and optimization of lead compounds in drug development. A lead compound in drug discovery is

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a chemical compound that has pharmacological or biological activity and whose chemical structure is used as an active site for chemical modifications to improve selectivity or pharmacokinetic parameters. Compounds identified by high-throughput screening usually have weaker affinities towards the target. The binding affinity and selectivity of these compounds are increased by suitable lead compounds [63]. One of the major challenges for ITC has been the identification of ligands with high binding affinities. ITC has been applied in drug design, where the binding affinities of the drug to the target were identified from thermodynamic parameters, such as free energy change (ΔG), enthalpy change (ΔH), entropy change (ΔS) and binding affinity constant (K_a) values. These thermodynamic parameters are related to each other by Eq. (5). For spontaneous binding between the drug and the target, ΔG is negative and is directly related to binding affinity.

High affinity between drug and target is achieved only when enthalpy and/or entropy contribute favorable energy. For a favorable binding between the drug and the target, the ΔH value is negative and ΔS value is positive. The favorable enthalpy is associated with the formation of hydrogen bonds and van der Waal's interactions; and the unfavorable enthalpy is associated with the desolvation of polar groups. The enthalpy associated with the desolvation of polar groups is ~8 kcal/mol [64], which is one order of magnitude higher than that of non-polar groups. For favorable enthalpy, the drug has to interact with the target by overcoming the unfavorable enthalpy resulted by desolvation. Therefore, the ΔH value represents the strength of the drug-target interaction relative to those with solvent. Ruben *et al*. [65] have reported the optimization of an antimalarial target, namely plasmepsin II inhibitors. In plasmepsin II inhibitors, a hydrogen bond donor is placed at different positions in a phenyl ring and the enthalpy change was studied by ITC. The hydroxyl group located at para position showed favorable enthalpy and this was considered the best position. On the other hand, by placing a methyl group at the same position results in an unfavorable binding enthalpy of 4.4 kcal/mol, which confirmed the best functional group at that position is a hydrogen bond donor. This shows that ITC is the powerful tool for identifying the most appropriate position for hydrogen bond donors or acceptors in the drug and target. Thermodynamics data for inhibitor binding to renin were derived by ITC [66]. The binding reaction between inhibitor and rennin was driven by favorable enthalpy changes (ΔH = –9.27 kcal mol^{-1}) but it was counterbalanced by unfavorable entropy changes (TΔS = –2.00 kcal mol^{-1}). When the entropy term was improved, there was a significant loss in the enthalpy term. Synthetic efforts were initiated by molecular alterations and finally the entropy term was improved by designing a compound by adding an additional electrostatic interaction, which had a favorable entropy term (TΔS = 0.43 kcal mol^{-1}) with an almost unchanged ΔH value. As a result the affinity of the optimized inhibitor was increased to about 45-fold compared with the initial compound.

Even though enthalpic interactions are required for extremely high affinity, the optimization of the binding enthalpy has been more difficult than the optimization of the binding entropy, the reason being that the enthalpy of desolvation of polar groups is very large and unfavorable [67]. In the binding thermodynamics of seven clinical protease inhibitors (indinavir, saquinavir, nelfinavir, ritonavir, amprenavir, lopinavir and atazanavir), the inhibitors (indinavir, nelfinavir, saquinavir and ritonavir) are dominated by a strong favorable entropy change [68]. The binding enthalpy is unfavorable for indinavir, nelfinavir and saquinavir, and only slightly favorable for ritonavir. Experimental results indicate that in the inhibitors, indinavir, ritonavir, saquinavir, and nelfinavir, the inhibitor/protein interactions are not strong enough to overcome the unfavorable enthalpy associated with the desolvation of protein and inhibitor group and the unfavorable enthalpy associated with the change in conformation of the protease upon binding. In all cases, favorable entropy contributes to the Gibbs energy. Two main factors contribute to the extremely favorable entropy change: a large favorable salvation entropy change that originates from the release of water molecules associated with the burial of a significant hydrophobic surface upon binding and a small unfavorable conformational entropy change. Therefore, entropic optimization is achieved by increasing the hydrophobicity of drug candidates and by introducing conformational constraints [69]. This favorable solvation entropy resulted from burial of hydrophobic groups. Protasevich *et al*. [70] studied the inhibitor affinity of four 2-(2-hydroxyphenoxy)phenol inhibitors, wherein the 4-position substituent varied from H to n-propyl to determine the contribution of the aliphatic chain to the binding to the wild-type (wt) enoyl-ACP reductase from *Escherichia coli* (FabI) and a drug-resistant mutant, (F203L)FabI, in which phenylalanine 203 is mutated to leucine. The inhibitor affinity to wt FabI and (F203L)FabI were increased with increasing aliphatic chain length, although the related affinity for (F203L)FabI was lower, and also, it showed no detectable binding to the 4-H inhibitor. The binding enthalpy became

less favorable with increasing aliphatic chain length. Therefore, the increase in affinity was due to favorable changes in solvation entropy. Hydrogen bond donor/acceptor functionality attached to a large aromatic ring can force part of the ring to be exposed to the solvent. This brings about a drop in the favorable desolvation entropy that partially or completely neutralizes any enthalpic gains due to hydrogen bond. In addition, when hydrogen bond donor and acceptor groups are stronger in the compound, these are hydrogen-bonded to water before binding. Therefore, the amount and type of hydrogen bonding is highly indicative of the specificity of the reaction. However, too many hydrogen donor groups on the drug molecule will cause low bioavailability due to poor cell membrane permeability. Conversely, less hydrogen binding results in poor specificity. Lipinski *et al*. [71] suggested that four parameters should be globally associated with solubility and permeability; namely molecular weight; Log P; the number of H-bond donors and the number of H-bond acceptors. The 'Lipinski rule of 5' states that: poor absorption or permeation are more likely when: there are more than 5 H-bond donors; the molecular weight is over 500; the Log P is over 5; and there are more than 10 H-bond acceptors. He also added that if two parameters are out of range, a poor absorption or permeability is possible. However, Li *et al* [72] reported in an analysis of the data in PDBcal that a diverse set of ligands with only 38% of the studies was obeying the Lipinski's Rule of Five. ITC data provide a way to optimize the number of hydrogen bonds with a minimum number of hydrogen bond donors and acceptors. Ernesto Freire [73] explained that for a highly favorable binding enthalpy it is not the number of polar groups present in the drug molecule that counts, but the quality of the hydrogen bonds that are made with the target protein. For example, two protease inhibitors, saquinavir and TMC-126, both have 11 polar atoms; however, saquinavir binds with unfavorable enthalpy (+1.5 kcal/mol) whereas TMC-126 binds with a highly favorable one (–12 kcal/mol) [74].

Biological systems are subject to enthalpy-entropy compensation, where the enthalpic gain from changing the structure of a compound to increase bonding interactions is offset by an entropic penalty, thus reducing the magnitude of change in free energy [75, 76]. The thermodynamics of the drug inhibitors acyclovir, ganciclovir, and 9-benzylguanine binding to human purine nucleoside phosphorylase (hsPNP) as a function of the substrate phosphate ion (Pi) concentration [77] showed that the binding reaction changed from an enthalpically driven reaction in the absence of Pi to an entropically driven reaction at 10 mM Pi. The dependencies of the driving-nature of the binding reactions on Pi concentration can be simulated by Pi binding to its catalytic site. However, the binding constants are unaffected by change in the bound Pi concentration because of enthalpy-entropy compensation. Burial of the apolar surface of small-molecule hydrocarbons is known to be accompanied by a favorable entropy change in aqueous solution due to the release of water molecules from the apolar surface into bulk. In contrast, desolvation of polar dipeptides is accompanied by the formation of intersolute hydrogen bonds or salt bridges, which is enthalpically favorable [78]. Extrapolating from this understanding of small-molecule thermodynamics, it seems that increasing polar surface area burial in a protein-ligand complex could be used as a strategy to provide a more favorable enthalpy of binding. However, in the context of protein-ligand interactions, the thermodynamics of the burial of a polar surface between proteins and ligands is affected by the influence of the detailed topography of the binding site and the balance between unfavorable enthalpy change due to dehydration and favorable enthalpy change due to the formation of protein-ligand hydrogen bonds [79].

ITC can also be applied to improve drug delivery systems. Chitosan has been extensively used as a biomaterial because of its immunostimulatory, antibacterial and antifungal activities [80, 81]. However, chitosan suffers from low solubility at a physiological pH of 7.4, which limits its use as an absorption enhancer in a neutral environment, *e.g*., for nasal peroral delivery systems. The solubility of chitosan was improved by using the derivatives of chitosan. The binding affinity of carboxymethylchitosan (OCMCS) with BSA was tested by ITC analysis [82]. ITC demonstrated that BSA bound to OCMCS with a molar ratio of 4.5:1. The binding isotherms appeared exothermic, which was ascribed to the contributions of H-bond and hydrophobic interactions.

3.3 ITC applications in cell metabolism

Isothermal microcalorimetry is currently used in the study of microorganisms, human and animal cellular systems, small animals, plants materials, and mammalian cells and tissues. Studies carried out in these areas have been published in Special Issues of *Thermochimica Acta* [83–85]. One important problem in studying cells using microcalorimerty is that the cultures may become anoxic due to limitations in the supply of oxygen [86, 87]. This problem has been solved using two

approaches. The first is to make the bioreactor the calorimeter (also termed macrocalorimetric technique) [88] and the second uses a combination of a bioreactor with a flow microcalorimeter [89]. This has a device to circulate the cells in suspension towards a microcalorimeter, which has a flow-through vessel (termed flow calorimetric technique). The principles and schematic representation of these different techniques have been extensively described elsewhere [90, 91].

The first macrocalorimetric technique, *i.e.*, dynamic calorimetry with a working volume of 10 L, was introduced in 1968 [92]. The major disadvantage arises from the brief interruption (5–9 min) of the cooling system, which resulted in relatively poor resolution especially for weakly exothermic or endothermic bioprocesses. The resolution of this system was further improved so that it had complete energy balance in a standard laboratory bioreactor with a working volume of 1.5 L without the need for disconnection of the cooling power [93]. Heat flow is calculated by mathematical models, to yield precise values.

Continuous calorimetry was developed using a 14-L laboratory bioreactor provided with a constant cooling power [94]. The reactor temperature was set using an electrical heater inside the reactor. Based on this principle, two commercially available reaction calorimeters were developed, the Bergh of Fermentation Calorimeter (BFK) [95, 96] and the Mettler-Toledo Reaction Calorimeter (RCl) [97]. The BFK type l has a 2-L working volume glass reactor and is double jacketed. The reactor temperature is monitored by a high resolution thermistor. A constant temperature difference is maintained by a silicone oil circulate. An advantage of the BFK-1 is that no corrections for heat losses through the reactor wall are required; however, this system could not provide sufficient cooling capacity for all applications, the thermopile wall is extremely fragile and the reactor design is impractical. For these reasons, a BFK type 2 was developed in which silicone oil is passed through the jacket of the housing to maintain the temperature at precisely that of the reactor contents, thus acting as an adiabatic shield.

Stockar's group [97] adapted a 2-L Mettler-Toledo RC1 heat accumulation calorimeter, capable of operating in isothermal, adiabatic or isoperibol modes. The detection limit for the reaction calorimeter was only ~2×10^6 cells/cm^3, generally an order of magnitude higher than the starting inoculum for animal cultures, which in a standard batch culture generally rise to a maximum concentration of approximately 1×10^6 cells/cm^3. A series of improvements to the Mettler-Toledo RC1 reaction calorimeter in terms of resolution in sterile operation, mixing and agitation, mass flows, and delivering air resulted in the detection limit sufficiently low to follow the course of batch cultures of many different types of animal cells [98, 99]. Two techniques for delivering air to the cell culture, *i.e.*, under surface aeration and purging by air, are investigated to study the consumption of glucose and the production of lactate [100]. The results indicated that the sparged culture was more aerobic and this resulted in less lactate and gave a lower $Y_{lac/glc}$ yield of 0.96 than those under surface aeration ($Y_{lac/glc} = 1.24$).

Garcia-Payo *et al.* [101] reported changes to the instrument in terms of limiting non-biological heat flows and altering the geometry of the glass reactors. The problems associated with this are direction of flow, slow maximum flow rate, narrow bore tubing and small volume of the measuring vessel. The Kemp research group developed a solution for measuring the heat flow rate of animal cell growing under the controlled conditions of the bioreactor [102]. It allows the measurement of the heat produced by circulating the cell suspension from the bioreactor to the flow calorimeter and then returning it to the bioreactor. The same research group also developed a new twin flow module calorimeter that is optimized for the use with cell suspensions as well as cells growing on microcarrier beads. The various fabricated instruments for flow calorimetery have been summarized in [91, 98]. Cultured animal cells, particularly those derived from either insect or mammalian tissues, are used increasingly in pharmaceutical industry. The heat produced by animal cells in culture can be used as the primary indicator of the kinetics of their metabolism [103]. Studies reported on Chinese hamster ovary cells (CHO320) genetically engineered to produce interferon-γ (IFN-γ) showed an improved formulation that indicates faster cell growth and greater specific rate (flux) of IFN-γ constitutive secretion, while decreasing glucose utilization and excretion of lactate that is toxic to cells.

Microcalorimetry was used successfully as a bioassay for the detection of the very small metabolic activation following binding of growth hormone to its cell surface receptor [104]. Activated metabolism and cell growth were observed (positive slop in heat flow) on the addition of trace amounts of added growth hormone (less than 25×10^{-12} mol/L). Information on the triggered cell division following the addition was also derived. Microcalorimetry can thus be considered a technique that can be used to test a vast number of therapeutically important substances on cells and tissues. Following the similar methodology, cell death was monitored on addition of anticancer

drugs [105]. The action of two anti-neoplastic drugs, methotrexate (MTX) and 6-thioguanine (6-TG), on a human T lymphoma cell line, CCRF-CEM, has been studied. The slope of the power-time curve after drug injection relative to that obtained during unperturbed growth was used to construct dose-response curves. Backman *et al.* [106] studied the growth and energy metabolism of cell with time by microcalorimetric study. The growth rate of cultured T lymphoma cells decreased with time. The changes could be attributed to the decrease in pH of the medium. Following these principles of studying cell death and cell growth by ITC analysis, this technique finds application in various fields, such as identification of some diseases, drug formulation and factors that affect the growth of a cell.

4 Conclusions

In this review we have presented recent trends and applications of ITC in bioseparation, drug development and studying the living organism. In addition, the methodology and analysis of the thermodynamic parameters derived have been described. In bioseparation, the mechanism of interaction has been elucidated from the thermodynamic parameters. The thermodynamic approaches serve as a framework for chromatographic separation. ITC application to drug development illustrate that binding affinities of the drug to the target can be identified from thermodynamic parameters. Improvements in terms of binding affinity and selectivity can be made using suitable lead compounds. The drug lead compounds are made by molecular alterations, and studying its affinity with the target by thermodynamic analysis by reaching an enthalpy and/or entropy to contribute favorable energy. ITC application in living organism can be used as the primary indicator of the kinetics of their metabolism and can be utilized in various fields especially in identification of some diseases and drug formulation.

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