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Applications of isothermal titration calorimetry - the research and technical developments from 2011-15.

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Review

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3 1 **Applications of isothermal titration calorimetry - the research and technical developments from**
4 2 **2011-15.**

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19 10 **ABSTRACT**

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21 11 Isothermal titration calorimetry is a widely used biophysical technique for studying the formation or
22 12 dissociation of molecular complexes. Over the last five years much work has been published on the
23 13 interpretation of ITC data for single binding and multiple binding sites. As over 80% of ITC papers are
24 14 on macromolecules of biological origin this interpretation is challenging. Some researchers have
25 15 attempted to link the thermodynamics constants to events at the molecular level. This review
26 16 highlights work done using binding sites characterised using x-ray crystallography techniques that
27 17 allow speculation about individual bond formation and the displacement of individual water
28 18 molecules during ligand binding and link these events to the thermodynamic constants for binding.
29 19 The review also considers research conducted with synthetic binding partners where specific binding
30 20 events like anion- π and π - π interactions were studied. The revival of assays that enable both
31 21 thermodynamic and kinetic information to be collected from ITC data is highlighted. Lastly published
32 22 criticism of ITC research from a physical chemistry perspective is appraised and practical advice
33 23 provided for researchers unfamiliar with thermodynamics and its interpretation.

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40 25 **INTRODUCTION TO RESEARCH BETWEEN 2011-2015**

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42 26 Research into isothermal titration calorimetry (ITC) started around 25 years ago as high-sensitivity
43 27 calorimetry instruments were developed. The publication of Ernesto Freire and coworkers' article
44 28 entitled "Isothermal Titration Calorimetry" in 1990 introduced this technique to researchers
45 29 interested in studying binding interactions.¹ Since 1990 there has been steady rise in research
46 30 publications on ITC (Figure 1) encouraged by the release of commercial instrumentation that made
47 31 this method accessible to a wide population of scientists. There are now around 600 to 700 peer-
48 32 reviewed papers containing research using ITC published annually and there are no signs of this
49 33 growth stopping. The field of protein chemistry has benefited most from ITC dominating the
50 34 published research though synthetic chemists have increasingly found ITC useful (Figure 2). Research
51 35 into lipids has used ITC to study demicellation with success and binding studies using nucleic acids,
52 36 carbohydrates and synthetic molecules are also represented.

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57 37 Despite the steady increase in research using ITC there have been no significant technical advances
58 38 in ITC instrumentation since 2010. Robotic automated instruments were already on the market in

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3 39 2010 and ITC can be considered a mature technology. There have been improvements in software
4 40 making the technology increasingly user friendly. The published ITC research is dominated by simple
5 41 one-site binding interactions where the mathematics and interpretation of the results are relatively
6 42 simple. ITC-based techniques like thermal analysis of enzyme kinetics,^{2,3} continuous ITC⁴ and protein
7 43 folding⁵ have received minimal uptake by the research community despite their apparent value.
8 44 There have been some recent advances in ITC-based techniques that are worth noting including
9 45 kinITC which collects kinetic and thermodynamic information for binding interactions,⁶ and advances
10 46 in ITC displacement assays for high-affinity binding reactions.^{7,8}

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14 47 The increased use of ITC to study binding interactions with synthetic molecules is worthy of note as
15 48 it provides highly defined molecules for binding studies. Protein binding studies have always been
16 49 complicated by the fact that many binding sites are not well characterised and the inherent flexibility
17 50 of protein molecules can make interpretation of binding site studies problematic. Progress has been
18 51 made on the interpretation of ITC data since 2010. The strengths and weaknesses of ITC are also
19 52 better understood. This knowledge however, has not uniformly trickled down to researchers
20 53 undertaking ITC analysis where presentation of ITC data and the interpretation of thermodynamic
21 54 parameters could be improved.

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24 55 A recent development has been the advent of the Journal of Visualized Experiments (JoVE). JoVE is a
25 56 PubMed-indexed video journal and ITC methods have been demonstrated by this journal.⁹⁻¹¹ This is
26 57 particularly useful for researchers unfamiliar with the practical applications of ITC and can form a
27 58 useful component in student or technician training.

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30 59 Between 2003 and 2012 the Journal of Molecular Recognition published annual reviews of ITC
31 60 research covering the years 2002 to 2010.¹²⁻²⁰ The authors John Ladbury, Ilian Jelesarov, Brett
32 61 Collins, Robert Falconer and their co-authors not only reviewed the literature but provided expert
33 62 advice on ITC use for the scientific community. The purpose of this current review is to appraise the
34 63 developments from the last five years since the last annual ITC review and provide advice on the
35 64 interpretation of ITC data. The author identified more than 2,500 articles reporting the use of ITC
36 65 between January 2011 and December 2015, after searching the Web of Science and Scopus
37 66 databases. This number of papers is impractical to cite in full so the author has selected
38 67 approximately 200 that he feels best represents the field and apologises for any resulting omissions.
39 68 These references have been classified into the following broad categories:

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43 69 (i) References cited in the introduction.¹⁻²⁰
44 70 (ii) Review and perspective articles.²¹⁻²⁹
45 71 (iii) Methods papers.³⁰⁻⁵²
46 72 (iv) Protein : protein interactions.⁵³⁻⁷⁰
47 73 (v) Protein interactions with other ligands.⁷¹⁻¹⁴³
48 74 (vi) Lipids, micelles and membranes.¹⁴⁴⁻¹⁵¹
49 75 (vii) Polysaccharides.¹⁵²⁻¹⁵⁵
50 76 (viii) Nucleic acids.¹⁵⁶⁻¹⁶⁹
51 77 (ix) Synthetic chemicals, polymers and nanoparticles.¹⁷⁰⁻²⁰⁷
52 78 (x) Enzyme kinetics.²⁰⁸⁻²¹⁷
53 79 (xi) Pre-2011 and non-ITC references.²¹⁸⁻²³⁴
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82 **INTERPRETATION OF SINGLE BINDING SITE ITC DATA**

83 Single binding site interactions are the simplest to study using ITC. If the c-value is between 1 and
84 1000 enough of the sigmoidal titration curve can be captured from which the stoichiometry,
85 disassociation constant (K_D), change in free energy (ΔG) and change in enthalpy (ΔH) can be directly
86 measured. From this the change in entropy (ΔS), can be calculated.²¹⁸ Note $c = M_0 / K_D$ where M_0 is
87 the initial concentration of the binding partner in the cell. Where the c-value is below 1 the
88 stoichiometry and change in enthalpy (ΔH) values are problematic and where the c-value is greater
89 than 1000 the disassociation constant (K_D) and change in free energy (ΔG) values are inaccurate. It is
90 also worth noting that the change in entropy value (ΔS) is calculated from the equation $\Delta G = \Delta H - T\Delta S$
91 and will contain any errors from both the ΔG and ΔH measurements. An excellent paper by Joel
92 Tellinghuisen written in 2012 provides further guidance for researchers designing ITC protocols to
93 generate precise thermodynamic data.⁴⁸

94 The first hurdle many researchers face is understanding the thermodynamic terms. While the
95 definitions for change in enthalpy and change in free energy definitions are fairly obvious and there
96 are some excellent text books on the subject.²¹⁹⁻²²⁰ The concept of entropy can be difficult to
97 comprehend. Entropy can be described as a measure of disorder within a system as well as the
98 energy state of a system.²²¹ For the interpretation of aqueous systems many authors rely on the
99 concept of entropy being the movement from ordered to disordered states (and vice versa) whereas
100 the idea of moving from a high energy state to a lower energy state is probably more accurate and
101 avoids the need to attribute structures to water that are questionable. The water around methyl
102 groups is an example where structural attributes have been used to describe water at the interface.
103 In the past these structures were described as being ice-like²²² and more recently they have been
104 described as clathrate-like cages²²³ or networks.²⁴ A simpler way of describing the water at the
105 interface with a methyl group is water that cannot hydrogen bond with the methyl group; this water
106 has a higher energy state than water surrounded by water where it can exchange protons freely. The
107 calculated entropy from ITC data is the sum of the entropies within the sample being studied and
108 will involve the ligand, its target, the water and any co-solutes (buffer, salts, etc.) within the sample.
109 This complexity makes it difficult to ascribe individual changes during binding (like displacement of
110 individual water molecules) to changes in entropy.²⁴ For further reading on the interpretation of
111 entropy that is written in a highly accessible manner try Frank Lambert's paper "A modern view of
112 entropy".²²⁴

113 The work using ITC to study drug candidates' interaction with drug targets has made researchers in
114 this field increasingly aware of the complexity that is occurring at drug binding sites. This has been
115 helped by the known crystal structure of some of the drug targets that were studied.²⁴ This enabled
116 speculation about the specific bond formation occurring and the displacement of specific water
117 molecules during binding.

118 During a binding interaction between a protein and a ligand the following occurs:

- 119 1. The ligand has to penetrate the protein's hydration layer (which may present an energetic barrier)
- 120 2. There is displacement of water from the part of the protein's and the ligand's surface where the
121 binding occurs (desolvation).

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3 122 3. There is also displacement of any co-solutes present at the protein surface. This is particularly
4 123 important where electrostatic interaction plays a role as charged co-solutes are often present at the
5 124 binding site and may need displacing.
6
7 125 4. Short-range bond formation (hydrogen bonding, van der Waal's interaction, pi-cation interactions,
8 126 etc.) between the protein and the ligand will occur.
9 127 5. There is the possibility of proton exchange between both binding partners and the buffer.
10 128 6. There is the possibility of conformational change of the protein; this is particularly important
11 129 where allostery plays a role in the protein's function.
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13 130 7. Finally there will be a rearrangement of the water adjacent to the ligand-protein interface.
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15 131 Each of these events during binding will have an effect on net ΔH and ΔS values.

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17 132 The role of the protein's hydration layer on binding interactions is contentious as the methods for
18 133 measuring this phenomenon like terahertz spectroscopy are still specialist techniques and not
19 134 familiar to most ITC users. There is evidence that a protein's hydration layer is more extensive and
20 135 complex than previously believed.²²⁵⁻²²⁷ It has also been shown that cosolutes can modify the
21 136 hydration layer.²²⁸⁻²²⁹ There is scope for future work using ITC in conjunction with low frequency
22 137 analysis of water.
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24 138
25 139 Gerhard Klebe's group used inhibitor binding to thermolysin^{24,76-77,108} to study the important
26 140 contribution of water displacement and rearrangement on the thermodynamics of inhibitor binding.
27 141 This was not a trivial undertaking. Firstly the structure was defined by x-ray crystallography at the
28 142 BESSY beamline in Berlin. This enabled the binding site to be well characterised and the possible
29 143 location of bound water molecules determined. In one study the ligands only differed in the
30 144 replacement of a methyl with a carboxyl group.⁷⁶ The difference in thermodynamics of binding was
31 145 attributed to the carboxyl group disrupting the water network around the filled binding site. A
32 146 second set of ligands were used with substitutions altering the ligands hydrophobicity. As the
33 147 thermolysin binding site is a hydrophobic pocket, the interaction would usually be considered as an
34 148 example hydrophobic interaction and would be entropy driven.⁷⁷ Interestingly, the addition of a
35 149 methyl group to the ligand resulted in an enthalpy-driven improvement in binding whereas addition
36 150 of further hydrophobicity to the ligand gave a predicted entropy-driven improvement. This was
37 151 ascribed to changes to the water at the surface of the protein ligand complex.^{77,108} The conclusion
38 152 from this research was that water played a minor role in the change in free energy but had a major
39 153 effect on change in enthalpy and entropy. The work did demonstrate our current inability to
40 154 consistently predict the thermodynamic profiles associated with relatively simple changes in ligand
41 155 structure even when the binding site was well characterised.²⁴
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48 157 The classical approach to the thermodynamics of binding would be to consider solvation as implicit
49 158 within the activity coefficients of the binding partners. Brian Castellano and Daryl Eggers argued that
50 159 for binding reactions in aqueous environments, the water should be treated as a coreactant. So the
51 160 binding equation was proposed that took water into account, $\Delta G^0 = -RT \ln K_i - [Q]_i \Delta G_i^{H_2O}$ where ΔG^0 is
52 161 the standard free energy constant, $[Q]_i$ is the concentration of the complex, K_i is the association
53 162 constant and $\Delta G_i^{H_2O}$ is the desolvation energy, all in a specific solution (i).¹⁷⁵ The example used was
54 163 calcium ion binding to EDTA conducted at different reactant concentrations and temperatures.
55 164 When $-RT \ln K_i$ was plotted against $[Q]_i$, the y-intercept gave the ΔG^0 and the slope the $\Delta G_i^{H_2O}$ values.
56 165 An observation was that K_i changes with concentration and that the $\Delta G_i^{H_2O} / RT$ had a near linear

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3 166 relationship to $1/T$. This research provides a method to determine values for the desolvation energy
4 167 associated with binding interactions.

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7 169 Displacement of co-solutes during binding is often overlooked during ITC studies. An example where
8 170 co-solute displacement was studied used metal cation binding to the synthetic p-
9 171 sulfonatocalix[4]arene (a ring structured molecule with four acidic sulpho groups where a metal ion
10 172 can bind).¹⁸¹ The presence of a counter ion such as sodium had a considerable effect on the
11 173 thermodynamics of binding. While p-sulfonatocalix[4]arene is a synthetic molecule the principle is
12 174 the same for proteins and other macromolecules where ions commonly interact with oppositely
13 175 charged constituent parts. Most ITC binding studies are in buffered solutions where the co-solutes
14 176 often comprise sodium chloride and a buffer that will interact with charged amino acid side chains
15 177 and affect the thermodynamics of any binding that involve electrostatic interaction. George
16 178 Whiteside's group used the pocket in human carbonic anhydrase II to examine the role of anions on
17 179 binding.⁹⁵ This work which combined ITC with x-ray crystallography and molecular dynamic
18 180 simulation suggested low charge density anions can associate with hydrophobic regions within the
19 181 binding pocket, altering the charge and water structure in and round the pocket.

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24 183 Proton exchange between either binding partner with the buffer received much attention before
25 184 2011.²⁰ A recent study of ligand binding to a t-RNA binding protein provided a good example of
26 185 proton transfer during ITC experimentation having a marked effect on change in enthalpy.¹¹⁹
27 186 Further analysis was able to identify which components of the binding partners were responsible for
28 187 the proton exchange with the buffer.

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31 189 The take home message is that interpretation of ITC data for binding interactions in aqueous
32 190 systems has to take displacement of water, co-solutes and protons into consideration. Commonly
33 191 used solutions like phosphate buffered saline contain the high-charge density phosphate anion
34 192 which binds relatively strongly to positive charged side chains and can interfere with ligand binding
35 193 to proteins (personal observation). Anyone considering selection of low charge density ions like
36 194 guanidinium hydrogen chloride or iodine to improve protein solubility would be advised to read
37 195 George Whiteside's paper before proceeding.⁹⁵ Chemicals like DMSO are commonly used to help
38 196 solubilise ligands that have low-solubility in water but the effect of DMSO on the binding partners
39 197 and their respective hydration layers has to be taken into consideration. The choice of the buffer and
40 198 other cosolutes to be used during ITC experiments is very important and needs careful
41 199 consideration.

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46 47 201 **INTERPRETATION OF MULTIPLE BINDING SITE ITC DATA**

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49 202 The study of proteins and protein complexes with multiple binding sites is of particular interest to
50 203 scientists interested in allosteric regulation where the binding of one molecule to a site affects the
51 204 binding of a second molecule to a separate site on the same protein or protein complex. Where the
52 205 two molecules are different (heterotropic allostery) this phenomenon is easily studied using ITC as
53 206 the ΔG , ΔH and ΔS values for the second binding event will be different for the protein with and
54 207 without the first molecule present. An example of heterotropic allostery is the formation of the
55 208 complex between mRNA containing poly(A) sequences with the translation factors polyadenylate-

209 binding protein-1 (PABP) and scaffolding protein eIF4G.¹³⁰ The ITC data gave clear evidence of
210 cooperative binding of eIF4G and Poly(A) to PABP.

211 Allostery can also occur where the multiple bind sites on the protein or protein complex bind the
212 same ligand (homotropic allostery). An example of this was the binding of acetyl coenzyme A to the
213 dimeric protein aminoglycoside N-(6')-acetyltransferase-li.⁹⁶ This study used a combination of ITC,
214 circular dichroism, and nuclear magnetic resonance spectroscopy to quantify the structural, dynamic
215 and thermodynamic aspects of allostery. The ITC binding isotherms are often non-sigmoidal due to
216 the different ΔG and ΔH values of the different binding events. Homotropic allostery presents the
217 challenge of calculating meaningful thermodynamic constants for the multiple binding sites³¹ and
218 for detecting positive and negative cooperativity.³⁴ While the mathematics for calculating ΔG , ΔH
219 and ΔS values for multiple binding sites has been determined and informative simulations have been
220 undertaken^{31,34} it is worth remembering that relatively small errors in the raw ITC data (especially
221 where few titrations are present for critical parts of the thermogram) can generate plausible but
222 misleading ΔG , ΔH and ΔS values for the binding sites.

223 Non-specific binding can be easily confused with multiple binding site interactions. There are many
224 molecules that will bind to proteins, nucleic acids and synthetic molecules, while not targeting
225 individual binding sites. Possibly the best studied family of molecules that bind "promiscuously" to
226 proteins are the polyphenolics.^{79,107,117,143} It is believed that polyphenolics hydrogen bond with the
227 peptide backbone of a protein. The complicating factor in studying non-specific binding of
228 polyphenolics to proteins is their propensity to cross-link proteins which can displace water around
229 the proteins and contribute to the recorded ΔG and ΔH values (personal observation). The ITC
230 binding isotherms are often non-sigmoidal and could be interpreted as evidence of allostery if cross-
231 linking was not taken into consideration.¹⁰⁷

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233 **METHODOLOGICAL ADVANCES**

234 **kinITC assay to capture both thermodynamic and kinetic information.** Burnouf et al 2012 proposed
235 a method for collecting both kinetic and thermodynamic data from ITC experimentation that could
236 be used for simple binding interactions and more complex processes.⁶ The example they studied
237 included the binding of the inhibitor Nevirapine to HIV-1 reverse transcriptase, and the binding of
238 thiamine pyrophosphate (TPP) to the *Escherichia coli* riboswitch present in the 5'-UTR of the thiC
239 mRNA which folded on binding of TPP. The paper's supplementary information provided details on
240 instrument response time, injection times and mixing times for their Microcal ITC200 which had to
241 be taken into account if this method was to be reliable. Work on the partial validation of kinITC used
242 surface plasmon resonance as the gold standard method for determining the kinetic on and off
243 constants. The collection of kinetic data using an ITC is obviously attractive as it does not require a
244 tether to a solid support but the assay must be well validated and the instrument response time,
245 injection times and mixing times for the instrument known.

246 The collection of both kinetic and thermodynamic data has also been applied to study RNA helical
247 packing.¹⁶⁶ By running the assay at different temperatures they were able to calculate the Arrhenius
248 activation energy and Eyring transition state entropy as well as the thermodynamic parameters for
249 GAAA tetraloop-receptor interaction in magnesium and potassium solutions.

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3 250 **ITC assays for the quantification of high-affinity binding interactions.** The standard ITC
4 251 displacement assay used to study high-affinity interactions has been around since 2000 and has
5 252 been used to study a range of high-affinity interactions.^{230,231} This technique uses the displacement
6 253 of a moderate-affinity ligand to lower the apparent affinity of a high-affinity ligand. A displacement
7 254 assay using weakly binding fragments to thrombin was run in parallel with direct (low-C) assay and
8 255 showed both methods yielded valid disassociation constants.⁴⁴ The direct low-C titrations, however,
9 256 have highly questionable stoichiometry. The displacement assay also had a drawback that different
10 257 displaced ligands affected the enthalpic values indicating that the choice of the displaced ligand was
11 258 important and that experimental conditions need to be standardised so comparison can be made
12 259 between different fragments. This phenomenon was ascribed to the solvation structure and protein
13 260 dynamics of the initial protein–ligand complexes before displacement occurred.⁴⁴ The displacement
14 261 method has a serious drawback as the high-affinity ligand of interest has to be soluble at high
15 262 concentrations (>100 μM). Many high-affinity drugs have low solubility in water making the
16 263 traditional displacement assay impractical. The competition assay published in Krainer et al 2012 can
17 264 be used to study low solubility high affinity ligands.⁷ In this assay the receptor was titrated into a
18 265 mixture of competing high- and moderate-affinity ligands which generated a biphasic isotherm that
19 266 was be used to quantify disassociation constants (K_D) and binding enthalpies (ΔH) for both ligands.
20 267 Another alternative approach was a single-experiment displacement assay.⁸ The assay involved the
21 268 titration of the high-affinity ligand into a solution containing the moderate-affinity ligand bound to
22 269 the receptor with excess moderate-affinity ligand. The isotherm was also biphasic and was used to
23 270 quantify K_D and ΔH values for both high-affinity and medium-affinity ligands competing for the same
24 271 binding site. This provides three different strategies for analysing problematic high-affinity binding
25 272 interactions.

26 273 **Software.** Researchers using ITC are recommended to appraise the software NITPIC (which claims to
27 274 be superior to Origin) and SEDPHAT that have been developed to assist in analysis of ITC data.^{39,45,52}
28 275 The program NITPIC can be downloaded for free from
29 276 <http://biophysics.swmed.edu/MBR/software.html>. SEDPHAT can be downloaded from
30 277 <http://sedfitsedphat.nibib.nih.gov/software> free of charge. AFFINImeter produce commercial
31 278 software that can be used for analysis of displacement assays, micellization experiments, kinITC, the
32 279 application of complex models for complex interactions, and ligand induced conformational change.
33 280 At the time of writing this software was only suitable for MicroCal data but they were intending to
34 281 release the software compatible with other brands of ITC.

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36 283 **SYNTHETIC MOLECULES**

37 284 Over 80% of research using ITC is with macromolecules of biological importance including proteins,
38 285 nucleic acids, lipids and carbohydrates. Macromolecules are poorly suited to studying specific
39 286 interactions. The use of ITC with synthetic molecules provides a range of opportunities to study
40 287 binding interactions using receptors that are simpler and well defined. This has enabled hypotheses
41 288 regarding interactions in aqueous solutions to be tested using well defined synthetic ligands. This
42 289 information can then be transferred to help our understanding of the interactions that are occurring
43 290 in proteins, nucleic acids, etc.

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3 291 There are several papers studying cation- π ; anion- π ; and π - π interactions. In one study ciprofloxacin
4 292 hydrochloride was used in an aqueous solution.¹⁹⁷ Ciprofloxacin hydrochloride has a quinolone ring
5 293 and a protonated amine. ITC was used alongside H^1 NMR spectroscopy to demonstrate one-
6 294 dimensional aggregates formed by π - π stacking and dimer formation brought together by cation- π
7 295 interaction. Anion- π were studied in aqueous solutions with a tren (tris(2-aminoethyl)amine)
8 296 molecule attached to a nitroso-amino-pyrimidine.¹⁷³ A range of anions were shown to interact with
9 297 the heteroaromatic ring. A large entropic contribution favoured association and was attributed to
10 298 displacement of water around the hydrophobic pyrimidine surface during association suggesting in
11 299 this case water displacement played an important contribution to this anion- π interaction. Anion- π
12 300 interactions were also studied using halides (Cl⁻, Br⁻, and I⁻) and “two-wall” calix[4]pyrrole
13 301 receptors with two six-membered aromatic rings in organic solvents.¹⁷⁰ The number and electron
14 302 drawing character of aromatic substitutions increased the positive electrostatic surface potential of
15 303 the centre of the six member ring enabling the anion- π interaction. The interaction of fullerenes to a
16 304 buckycatcher (comprised of two corannulene subunits tethered together) in a range of organic
17 305 solvents is an example of binding with a strong π - π interaction component.¹⁸⁹ In a binding
18 306 interaction where solvent displacement played a significant role, the change in entropy played a
19 307 minor role in driving binding which surprised the authors.

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25 308 A synthetic octa-acid host with a hydrophobic pocket was used to study the effect of anions on
26 309 binding of small molecule ligands.¹⁸² In the case of low charge density anions like ClO₃⁻ the anion
27 310 was found to enhance affinity at low concentrations and weaken it at high concentrations. At higher
28 311 ClO₃⁻ concentrations, for the small molecule ligand to bind to the hydrophobic pocket required
29 312 displacement of the anion. This supports the theory of Kim Collins that explains the behaviour of low
30 313 charge density anions and protein solubility in terms of low charge density anion interaction with
31 314 hydrophobic surfaces on the protein.²³² While the synthetic octa-acid host study is ongoing it does
32 315 provide the opportunity to challenge or confirm the theories for low charge density anion
33 316 interaction with hydrophobic pockets at a nanometer-scale and complements work undertaken with
34 317 binding to proteins in the presence of low charge density anions that observe similar effects.⁹⁵ It also
35 318 has the capacity to challenge theories about the activity of medium and high charge density anion
36 319 indirect interaction through competition for solvent.²³³

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41 320 In an interesting study, allostery was mimicked using a dual-cavity basket which had six alanine
42 321 residues at the entrance of two juxtaposed cavities that was designed to trap organophosphorus
43 322 nerve agents.¹⁷⁷ Molecular dynamic simulation and H^1 NMR spectroscopy suggested a negative
44 323 homotropic cooperativity of binding in water. This is an attractive candidate for ITC studies as it
45 324 could be used to validate computer simulations of negative cooperativity binding.

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49 326 CAUTIONARY NOTE

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52 327 In 2015, Brian Pethica from Princeton University wrote a highly critical paper entitled “Misuse of
53 328 thermodynamics in the interpretation of isothermal titration calorimetry data for ligand binding to
54 329 proteins”²⁶ which should serve as a cautionary note for scientists who don’t have a strong
55 330 background in thermodynamics. Pethica’s critique, however, should not dissuade researchers from
56 331 using ITC to study binding as long as they are aware of the assumptions that predicate the
57 332 calculation of the thermodynamic constants.

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3 333 The first key assumption behind the equation $\Delta G = \Delta H - T\Delta S$ (where ΔH is the change in enthalpy, ΔG is
4 334 the change in free energy and ΔS is the change in entropy) is that the binding reaction is reversible
5 335 and that equilibrium has been reached. This assumption is acceptable for most binding reactions but
6 336 it should be remembered that allosteric change in a binding partner could prevent the ligand
7 337 returning to the solution. The most common error in published ITC data was too shorter time
8 338 between titrations which does not allow the peak to return to the baseline (i.e. equilibrium was not
9 339 reached before the next injection) and this key assumption was not met.

12 340 The second assumption is that the ligand and the macromolecule (protein, nucleic acid or synthetic
13 341 molecule) are totally soluble. In practice many ligands such as drug candidates have low solubility in
14 342 water. In some cases ligand preparations may include insoluble along with the soluble ligand. When
15 343 injected into the ITC cell some of the insoluble material will dissolve and there will be a ΔH
16 344 associated with this event. The use of control titrations of ligand into buffer (without the
17 345 macromolecule present) and titrations of buffer (without the ligand) into the macromolecule can be
18 346 used to detect this type of event occurring. The use of these controls should be a normal part of ITC
19 347 experimental design.

23 348 The third assumption is that macromolecule solutions are ideal (i.e. there are no macromolecule-
24 349 macromolecule interactions, no macromolecule-cosolute interactions, and no interactions between
25 350 macromolecule-ligand complexes). Macromolecule solutions are not ideal. Cosolutes interact with
26 351 macromolecules both by direct binding and indirectly by modifying their hydration layers.²³³⁻²³⁵
27 352 Macromolecules similarly interact with each other or compete with each other for water for their
28 353 hydration layers.^{226,229} The issue of cosolutes altering the thermodynamics of ligand binding is
29 354 unavoidable and the researcher has to accept that the thermodynamic constants derived from their
30 355 research are for the solution conditions used and will change if different buffer, pH or temperatures
31 356 are used. The issue of macromolecule-macromolecule interactions is also unavoidable. Even a target
32 357 like EDTA demonstrated concentration-dependant thermodynamics of binding to calcium ions.¹⁷⁴
33 358 This was attributed to the desolvation of the binding partners and demonstrated that undertaking
34 359 ITC at several target concentrations will provide a better understanding of the non-ideality of
35 360 macromolecule solutions.

36 361 To overcome the criticism from physical chemists like Brian Pethica, the author recommends that
37 362 researchers should do the following:

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- 39 364 • Outline the assumptions behind the thermodynamic calculations in their papers.
 - 40 365 • Make sure titration peaks do reach the baseline (achieving equilibrium).
 - 41 366 • Run the control titrations of ligand into buffer (without the macromolecule present) and
42 367 buffer (without the ligand) into the macromolecule as a standard part of the ITC
43 368 experimentation then present these thermograms in the paper or as supplementary
44 369 information.
 - 45 370 • Specify the conditions used for the binding experiments including the composition of both
46 371 titrant solution and the solution in the sample cell (include pH and temperature). Also include
47 372 the specific titration strategy used. While this does not avoid non-ideality of macromolecule
48 373 solutions it does define the experimentally derived thermodynamic constants for the precise
49 374 conditions used.
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3 374 • For experimentation with low solubility ligands, be careful that the ligand is totally dissolved
4 375 and if chemicals like DMSO are used to improve ligand solubility, consider their potential
5 376 interaction with the binding partners.
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9 378 **REQUEST FOR RAW DATA PUBLICATION**

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11 379 The author would like to suggest that editors and reviewers of articles containing ITC data should
12 380 request that the raw ITC data (the experimentally derived thermograms) should be published in the
13 381 paper or as supplementary material. There are many ITC papers where the calculated binding
14 382 isotherms alone are published without the experimentally derived thermograms. To the experienced
15 383 ITC operator the raw data contains a wealth of information and should be provided to verify that the
16 384 analysis was done to a high standard. The raw data can confirm that the baseline was steady and
17 385 equilibrium was reached before the next injection. The raw data can also be used to better
18 386 understand the kinetics of the interaction and detect mixed interactions (e.g. rapid binding followed
19 387 by slow aggregation). It is the author's opinion that much useful data is being lost and that
20 388 confidence in published data is eroded due to the frequent failure to publish raw ITC data.
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26 390 **Figure Titles**

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28 391 Figure 1 Articles written with isothermal titration calorimetry content since 1990 sourced from the
29 392 Web of Science™.

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31 393 Figure 2 Subject material studied using isothermal titration calorimetry in 2014. Note protein related
32 394 research accounted for 67% of the articles. Synthetic compounds were 17%, lipids and micelles were
33 395 6%, nucleic acids were 4%, carbohydrates were 3% and the remainder were 3% of the articles.
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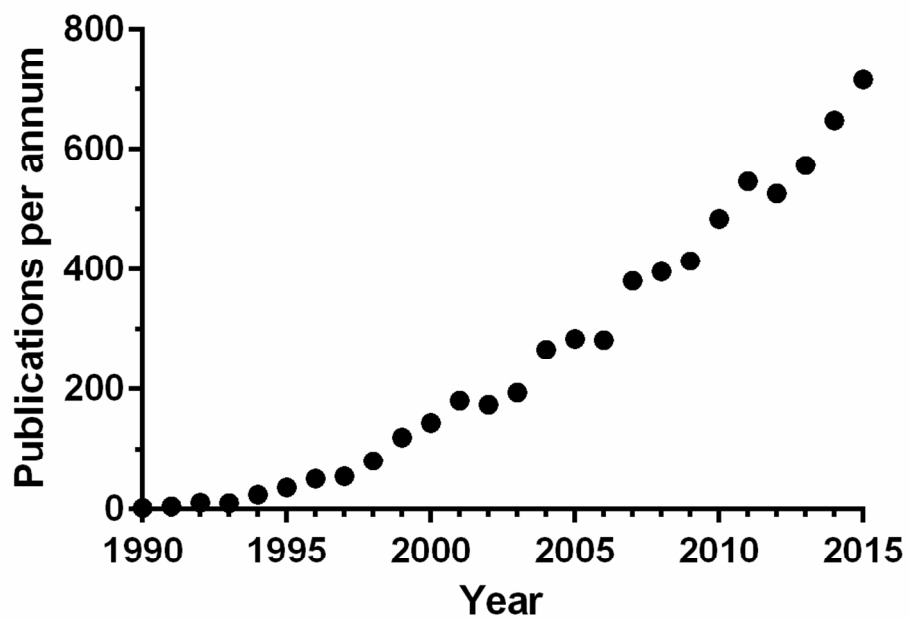
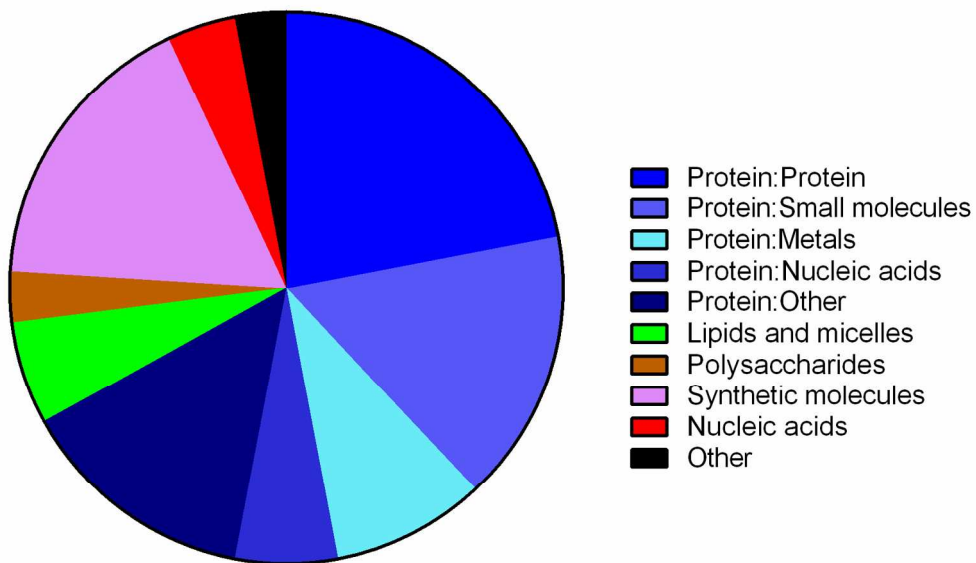


Figure 1
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154x90mm (300 x 300 DPI)

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