JOURNAL OF APPLIED BIOANALYSIS, July 2016, p. 90-102.
http://dx.doi.org/10.17145/jab.16.013 (ISSN 2405-710X)
Vol. 2, No. 3

OPEN ACCESS

REVIEW

Biophysical characterization of antibodies with isothermal titration calorimetry

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(Received: 3 June 2016, Revised 30 June 2016, Accepted 5 July 2016)

Antibodies play a key role in the immune response. Since antibodies bind antigens with high specificity and tight affinity, antibodies are an important reagent in experimental biology, assay development, biomedical research and diagnostics. Monoclonal antibodies are therapeutic drugs and used for vaccine development. Antibody engineering, biophysical characterization, and structural data have provided a deeper understanding of how antibodies function, and how to make better drugs. Isothermal titration calorimetry (ITC) is a label-free binding assay, which measures affinity, stoichiometry, and binding thermodynamics for biomolecular interactions. When thermodynamic data are used together with structural and kinetic data from other assays, a complete structure-activity-thermodynamics profile can be constructed. This review article describes ITC, and discusses several applications on how data from ITC provides insights into how antibodies function, guide antibody engineering, and aid design of new therapeutic drugs.

Keywords: protein engineering, biopharmaceutical, thermodynamics, isothermal titration calorimetry, antibodies.

Introduction

Antibodies, also known as immunoglobulins, are naturally produced glycoproteins, secreted by plasma cells derived from B cells. Antibodies have two distinct functions: to specifically bind to their target antigens, and to elicit an immune response against the bound antigen by recruiting other cells and molecules. Antibodies are classically "Y-shaped" and each tip of the Y contains a binding region that is specific for an antigen (antigen-binding fragment or Fab). The stem of the Y is the fragment crystallization (Fc) region, which interacts with Fc receptors to initiate effector function.

The association between an antibody and antigen involves a network of non-covalent interactions between the epitope (the binding site on the antigen), and the paratope (the binding site on the antibody). The ability of antibodies to bind antigens and receptors with a high degree of specificity and tight affinity is the key to the immune response. Specificity and affinity are qualities that make antibodies an important tool in experimental biology, assay development, biomedical research, diagnostics, vaccine production, and as therapeutic drugs. The diversity of antibody-binding capabilities is striking, since there is a high degree of structural similarity between antibodies. Recombinant antibodies, antibody engineering, thermodynamics and structural data have provided a deeper understanding of how antibodies function and recognize antigens and receptors.

The development of monoclonal antibody technology in the late 1970s and early 1980s opened a new era in therapeutics through the production of monoclonal antibodies, which are specific for a single epitope of an antigen [1]. Antibodies are attractive as drugs due to their high degree of target specificity, as well as the antibody's distinct structural and functional domains. Early engineering work concentrated primarily on "humanizing" the antibody, making it tolerable to the human immune system. The modification of antibodies via engineering is of major interest by biopharmaceutical companies since changes in antibody functionality and physicochemical

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properties will broaden their application area. [2,3,4].
Protein engineering is also creating novel antibody formats, as well as antibody-drug conjugates. When an antibody is designed as a drug, its immunogenicity, affinity, stability, effector functions, half-life, and tissue penetration and distribution should be taken into consideration and optimized accordingly. Therapeutic antibodies have mainly utilized the IgG1 subclass, and have been developed to treat a large range of diseases such as cancer, autoimmunity and inflammation. Antibodies are also used in the development of vaccines against targets such as HIV-1.

Characterization of antibodies

Biochemical and biophysical assays are used to understand how native and recombinant antibodies (including therapeutic antibodies) function, by measuring affinity/bioactivity, kinetics and thermodynamics of binding, the mechanisms of recognition, and structure-activity relationships. These assays include enzyme-linked immunosorbent assay (ELISA), radioiummnoassays, western blots, immunoprecipitation, fluorescence spectroscopy, analytical ultracentrifugation, equilibrium dialysis, surface plasmon resonance (SPR), biolayer inferometry (BLI), microscale thermophoresis (MST), circular dichroism, x-ray crystallography, nuclear magnetic resonance (NMR) spectroscopy, mass spectrometry, differential scanning calorimetry (DSC) and isothermal titration calorimetry (ITC). These assays are also used to develop and characterize antibody drugs during discovery, development, and production. The binding affinity of an individual antibody-antigen interaction needs to be specifically defined by precise measurement of its dissociation constant, K_D. The smaller the K_D the tighter the binding affinity, and the comparison of the K_D values is an important component of binding identification, assessment of binding selectivity, and specificity. Several assays are used to measure K_D of antibody-antigen interactions, including ITC.
Simply knowing the binding affinity is not sufficient for a complete understanding of the antibody-antigen interaction, however. A full thermodynamic analysis provides information related to the molecular forces at work between the antibody and antigen, or between the antibody and receptor. ITC is the only assay able to provide a direct measurement of the binding enthalpy, as well as the binding stoichiometry and the affinity of the antibody-antigen interaction, or the interaction between the antibody drug and its target. Data from ITC provides insights into how antibodies function, and can aid in the engineering of antibodies and the design of new therapeutic drugs. When thermodynamic data are used together with structural and kinetic data from other assays, a complete structure-activity-thermodynamics profile can be constructed. This review article describes ITC, and discusses several applications on how ITC is used to characterize antibodies.

Introduction to isothermal titration calorimetry (ITC) and binding thermodynamics

ITC directly measures the heat change associated with binding events. ITC determines the binding affinity (or K_D the dissociation constant) for protein-protein, protein-drug, protein-carbohydrate, or any biomolecular interaction. ITC data are used in life science basic research, as well as during the discovery and development of small molecule drugs and biopharmaceuticals. Modern ITCs measure K_D values in the millimolar (weak affinity) to single digit nanomolar (tight affinity) range. Binding enthalpy, entropy, heat capacity, and binding stoichiometry (number of binding sites) can also be measured from

ITC [5-11]. ITC is established in life sciences as the "gold standard" method to study binding processes, since it is label-free, in-solution, sensitive, versatile, and can be used with different biomolecules in a variety of buffers. Since ITC measures heat change, it is a universal detection system. This means native proteins can be used, and there is no need for any kind of label, tag, or marker. Unlike SPR, ITC is performed with binding partners in solution, and does not require immobilization or chemical modifications of the interacting materials. These chemical modifications can potentially interfere or affect the binding interaction under study. SPR assays require less protein and have faster throughput, ITC is commonly used to validate binding affinity measurements from SPR.

ITC does not require any fluorescent measurements, so is not susceptible to potential artifacts as other fluorescence-based assays. ITC has no molecular weight limits, and can be used with any size molecule, such as antibodies binding of small molecular weight antigens, like carbohydrates, as well as interactions with large proteins like immunoglobulin complexes. ITC is the only direct measuring for binding enthalpy - other binding assays, like fluorescence and SPR, determine van't Hoff enthalpy, which is a calculated value determined by the temperature dependence on the K_D. ITC also directly determines the binding stoichiometry of an interaction, which is useful in confirmation of protein activity and specificity.

The first commercially available ITC was the OMEGA ITC, launched in 1987, from MicroCal [12]. Modern ITCs (including MicroCal PEAQ-ITC system from Malvern Instruments [13], and Affinity ITC from TA Instruments [14] are "power compensation" ITCs, which measure the temperature difference between the reference and sample cells, and change the thermal power compensation to bring the temperature difference back to zero. The power compensation is directly related to the binding heat.

Figure 1A represents how ITC works. One binding partner (the "ligand") is placed in the ITC injection syringe, which is titrated into the sample cell and mixed with the second binding partner (the "macromolecule"). Typically, the antibody is placed in the ITC cell, and the antigen in the ITC syringe, however ITC can be done with the reverse experiment. Both binding partners are in the identical buffer. The matched reference cell is filled with water. The ITC is programmed to inject a specific volume of ligand into the macromolecule solution, at timed intervals. The injection syringe also has a paddle in the bottom, so the mixture is stirred throughout the experiment. **Figure 1B** is a representation of the raw output of an ITC experiment. In this example the binding heat is exothermic – the negative deflection of the heat change represents heat given off with binding of ligand to macromolecule. Each peak represents the heat change of a single injection of ligand. The heat change evolved (or absorbed) during an ITC titration is proportional to the fraction of bound ligand. The first injections are large because most of the injected ligand is binding to the macromolecule. As the macromolecule becomes saturated with increasing ligand concentration, less of the ligand will bind and therefore less heat is generated for later injections. At the end of the experiment very little ligand is bound and the small heats are due to the heat of dilution. In this example the experiment used 19 injections for completion.

When the raw ITC data are uploaded into the ITC data analysis software, an integration baseline is automatically generated and the areas of each peak are calculated and then plotted as a function of the molar ratio of ligand to macromolecule in the cell. This is shown in **Figure 1C**. The black dots represent data points of individual peaks. The red line is the best fit to a binding algorithm from which we determine the enthalpy of the interaction, the stoichiometry (related to the midpoint of the curve)

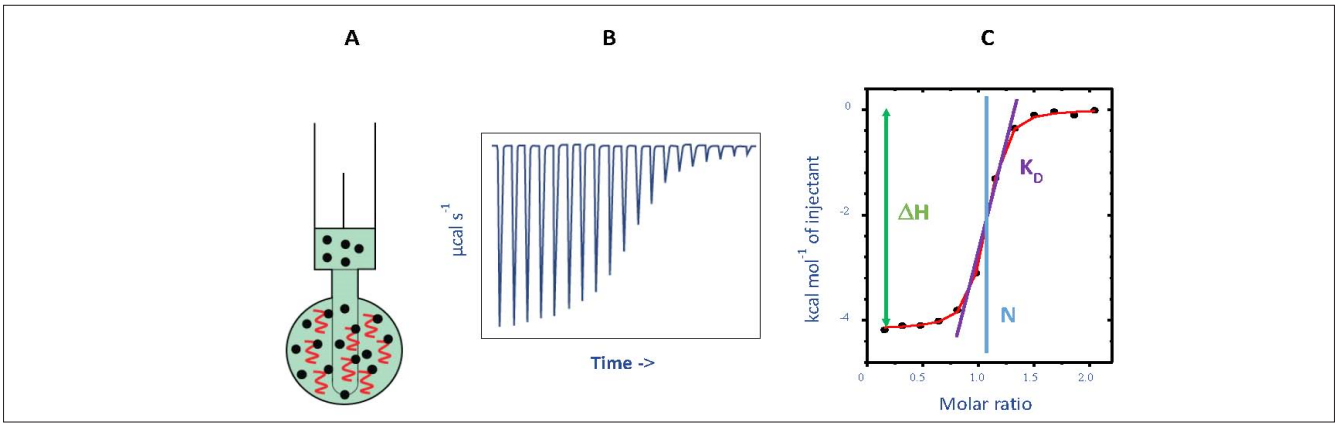

Figure 1. How ITC works. A: Schematic of ITC cells and injection syringe. B: Representative raw ITC data from titration experiment. C: Representative binding isotherm from ITC experiment, fit to one set of sites binding model.

and the K_D (related to the concentration and the sigmoidocity of the data). For antibody-antigen interactions, the stoichiometry is typically 2 moles of antigen binding one mole of antibody.

ITC accurately measures K_D from millimolar to nanomolar values. ITC is used as the primary K_D assay, as well as a secondary assay to validate K_D values from other assays such as SPR and ELISA. For tight affinities (K_D in the single-digit nanomolar range or lower) there are modified ITC procedures (see below).

The K_D is related to the standard free energy change ($\Delta G°$) of the interaction by the Gibbs equation **Equation (1)**:

$$\Delta G° = RT\ln K_D \quad (1)$$

Where R is the gas constant and T is the temperature of the reaction (in K). $\Delta G°$ needs to be negative for a process to occur. The free energy value allows a direct comparison of the affinities of interactions at a specific set of conditions (temperature, buffer, pH, etc.). The tighter the binding affinity, the smaller the K_D, and $\Delta G°$ becomes more negative. Knowing the K_D from ITC, one can determine the free energy change from **Equation (1)**.

$\Delta G°$ is also related to the binding enthalpy ($\Delta H°$) and binding entropy ($\Delta S°$) by **Equation (2)**:

$$\Delta G° = \Delta H° - T\Delta S° \quad (2)$$

Using **Equation 2**, since the free energy and enthalpy changes are known from ITC data analysis, we can determine the change in entropy.

Characterization of binding enthalpy and entropy provide insight into the binding mechanism. Several binding reactions can have similar affinities and free energy changes, however enthalpy and entropy contributions can vary for the for the different binding interactions. Enthalpy change is the net heat change associated with making and breaking of non-covalent bonds (including hydrogen and ionic bonds, and van der Waals interactions). When there is a net increase in non-covalent interactions of the bound complex, compared to the individual molecules, that is a favorable enthalpic process.

On a basic level, entropy is a measurement of the tendency for a system to "disorder." When two biomolecules bind to each other, that interaction can result in a more ordered (entropically unfavored) or disordered (entropically favored) state, when compared to the two individual biomolecules. Increased order arises when there are restrictions on the rotational and translational degrees of freedom at the binding interface. Increased disorder is from the release of water from a binding region into the bulk solvent (solvation entropy). A major contribution to the entropic contribution for binding is the hydrophobic effect, associated with the burial of non-polar (hydrophobic) groups away from the solvent, resulting in "freeing" water molecules.

Figure 2 represents the thermodynamic parameters of three interactions with the same K_D, for example three recombinant antibodies binding to the same antigen. All three experiments have the same free energy change. For this graphic representation, all the "favorable" thermodynamic parameters are represented by a negative value, and unfavorable contributions are positive values. For the experimental data in **Figure 2A**, enthalpy is favorable (negative) meaning binding is driven by formation of hydrogen and other non-covalent bonds. Entropy is unfavorable suggesting there is a loss of conformational freedom associated with binding. The data in **Figure 2B** has a favorable entropy value, suggesting binding occurs via the hydrophobic effect, and an unfavorable enthalpy suggesting that no new hydrogen bonds are formed during the interaction. The thermodynamic data in **Figure 2C** has both favorable enthalpy and entropy contributions.

"Enthalpy-entropy compensation" is a phenomenon which has been observed in binding studies with proteins and other biomolecules [15]. This is shown by plotting the enthalpy values versus the entropic value for a series of binding interactions (for example, binding of an antigen to wild type and engineered antibodies) and observing a linear relationship. This means when the enthalpy becomes more favorable (more negative) there is a com-

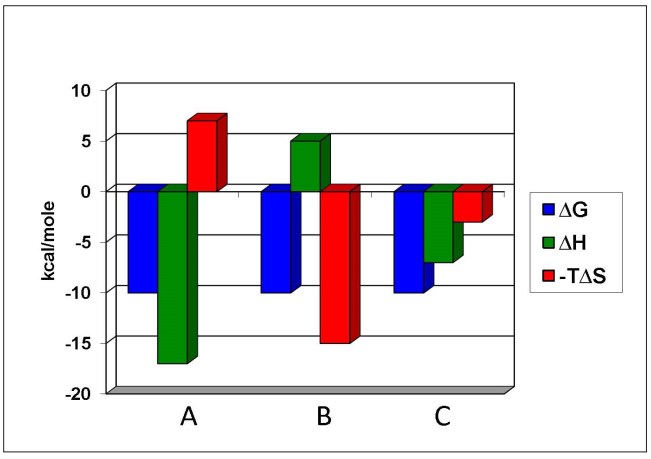

Figure 2. Representative thermodynamic data from ITC. ΔG data are blue bars, ΔH data are green bars, and $-T\Delta S$ data are red bars. A: Binding with favorable enthalpy. B: Binding with favorable entropy. C: Binding with favorable enthalpy and entropy.

pensatory change in the opposite direction of the entropic contribution, with a net result of little or no different in free energy or binding affinity.

The observed enthalpy from ITC not only includes binding heat, but also contributions from conformational changes, and heats of ionization of the buffer and the biomolecules. For thermodynamic characterization, it is important to specifically define the buffer composition of the ITC experiment, since enthalpy (as well as binding affinity) can be affected by buffer composition and pH. It is also important to conduct well-defined control experiments to account for other possible contributions.

It is well-established that interactions involving proteins show pH and buffer dependence, due to "proton linkage." If there is a change in the protonation state of either binding partner, enthalpy from ITC experiments will vary for different buffers, depending on the buffer ionization heats [16,17]. The heats of ionization of common buffers are known, making it possible to determine the number of protons absorbed or taken up by the binding event, as well as the intrinsic enthalpy (in absence of any buffer effect).

ITC should be performed over a range of different temperatures, and by plotting temperature versus observed enthalpy one can determine the change in heat capacity ($\Delta Cp°$) associated with the binding **(Equation 3)**:

$$\Delta Cp° = d(\Delta H)/d(T) \quad (3)$$

The heat capacity change is frequently negative (that is the enthalpy change is more negative at higher temperatures), when the bound complex is considered the "reference state." This arises due to the correlation between heat capacity change and changes in surface hydration associated with binding, causing burial of surface area [15]. If temperature versus enthalpy plot reveals a non-linear (temperature dependent) heat capacity change, that suggests other possibilities including unfolding/refolding of protein at higher temperatures, or coupled equilibria besides binding.

As mentioned above, the only way to directly measure the enthalpy of a binding reaction is determining the calorimetric enthalpy from ITC. It is possible to calculate thermodynamic parameters from other binding experiments where one can measure a K_D value (for example SPR or fluorescence) at several temperatures and using the van't Hoff analysis plot $\ln K_D$ versus 1/T. If this plot is linear the van't Hoff enthalpy can be determined from the slope [15]. However, for most binding interactions involving proteins, the van't Hoff analysis plot is not linear due to temperature dependence of the enthalpy change and heat capacity change. It is possible for some binding reactions to have similar calorimetric enthalpy (by ITC) and van't Hoff enthalpy (by SPR) [18] however frequently the values differ, so ITC is recommended whenever thermodynamic analysis is critical.

A complete thermodynamic profile of a binding interaction by ITC requires measuring free energy, enthalpy, and entropy at a defined temperature and buffer composition, and looking at the heat capacity change if there is a temperature dependence on the enthalpy. Different buffers and pHs should also be used to see if there is any proton linkage. For more detailed discussions of thermodynamics and ITC, see references [7-11,15,19].

ITC does use more protein compared to other biophysical and biochemical assays, and for best results purified materials are recommended. Recent advances and design improvements resulted in modern instruments which are more sensitive and use less protein compared to early ITCs, making ITC easier to use and more cost-effective [20]. Older ITCs needed 2 milliliters (or more) of protein sample to fill the ITC reaction cell. A newer instrument like MicroCal PEAQ-ITC (Malvern Instruments) has a 200 microliter cell volume, and requires 280 microliters of sample volume to fill the ITC cell without bubbles. For an antibody solution at recommended concentration range by the instrument supplier (3-20 micromolar) that would mean using 150 to up to 1000 micrograms of purified antibody per ITC experiment. Since ITC is nondestructive, it is also possible to recover the precious protein after the experiment, and use for another experiment or for crystallization.

Binding affinity and thermodynamics of antibody-antigen binding

Antibody binding to its antigen is a basic step for the immune response. Antigen-antibody complexes come from noncovalent interactions, including electrostatic and van der Waals forces, hydrogen bonds, and hydrophobic effects [10]. The interaction between a specific antibody and antigen is controlled in terms of thermodynamics, and investigations of thermodynamic properties, as well as structure, are crucial for understanding the nature of molecular recognition by antibodies [21-23].

Thermodynamic parameters are closely related to the particular antibody's mechanism of interaction, which usually includes formation of an encounter complex and stabilization steps. To select and engineer antibodies for therapeutic and diagnostic purposes, understanding these parameters is crucial. Antibody-antigen interactions were among the experiments performed with early versions of ITC instruments [24-26] For example, Raman et al.

[24] looked at the interactions between horse heart cytochrome c with two monoclonal antibodies, which recognized different epitopes on cytochrome c. The K_Ds were too tight to directly measure by ITC, so they determined binding affinities by other assays and used these values for the thermodynamic analysis. ITC experiments showed binding enthalpy and entropy for the two antibodies binding to cytochrome C were similar at 25 °C. However, performing ITC at different temperatures and in different buffers, showed different ΔCp° values and proton linkages for the two antibodies binding to cytochrome c. One antibody bound to cytochrome c with a large negative ΔCp° and a net uptake of one proton, while the second antibody bound to cytochrome c with a smaller ΔCp° and no protonation changes. This showed the two antibodies bound by different mechanisms.

Akiba and Tsumoto [27] discussed the role of thermodynamics in antibody-antigen interactions, by using mutation analysis of antibody variable regions. Antibodies against hen eggwhite lysozyme (HEL) are a useful model case for interaction with a protein antigen. Several studies have determined the effects of amino acid mutations of anti-HEL antibodies [28-30].

One project looked at the role of hydrogen bonding of antigen-antibody complexes with the interaction between HEL and its HyHEL-10 variable domain fragment (Fv) antibody [31]. They constructed three antibody mutants and investigated the interactions between the mutant Fvs and HEL. ITC results indicated that the mutations significantly decreased the negative enthalpy change, despite some offset by a favorable entropy change. Wild-type and mutant antibodies showed similar ΔCp° values, X-ray crystallography also showed that the antibody-antigen complexes for the different mutants had nearly identical structures, including the positions of the interfacial water molecules. These results suggested that hydrogen bonding via interfacial water enthalpically contributed to the Fv-HEL interaction, and hydrogen bonding "stiffened" the antigen-antibody complex.

Thermodynamics, in conjunction with kinetic and structural analyses of mutant antibody-antigen complexes, provide fundamental information about the formation of complexes in terms of the contribution of individual amino acids. In the case of anti-HEL antibodies, investigations on the thermodynamic parameters have revealed different roles of aromatic, charged and hydrogen bond-forming polar amino acids [27]. The thermodynamic properties of each amino acid in the antibody-antigen interface, in combination with the structure, give insight to the characteristics of the respective antibody-antigen complexes.

Characterization of tight binding affinities by ITC

The lowest K_D value (tightest binding affinity) that can be directly measured by ITC is in the single-digit nanomolar range [5-11]. ITC data for tighter affinities (single digit nanomolar and lower) cannot be precisely determined from a single ITC titration experiment, since the binding will be saturated within one or two injections, and there are not enough data points to calculate the K_D from the binding isotherm. For high affinity interactions, the displacement titration method (also called competitive ITC) involves artificially lowering the apparent affinity of the strong ligand by adding a weaker ligand as a competitor [32]. Aweda and Meares [33] used the displacement method for looking at antibody 2D12.5, which bound yttrium S-2-(4-aminobenzyl)-1,4,7,10-tetraazacyclododecanetetraacetate, and they achieved reproducible single-digit nanomolar K_D measurements.

Effect of antibody glycosylation to binding activity

It is well-established that all IgG subclasses have a single N-linked glycan in the Fc, which is essential for IgG effector functions [34,35]. Each immunoglobulin class also has a unique glycosylation profile, which contribute to its activity, function and stability. The properties of a series of normal, truncated and aglycosylated glycoforms of IgG1-Fc, generated *in vitro*, were subjected to a series of structural biology and biophysical assays, including x-ray crystallographic analysis, stability studies with DSC and Fcγ receptor binding with ITC and SPR [36]. DSC of two glycoforms exhibited two transition temperatures, representing the unfolding of the C_H2 and C_H3 domains. Sequential removal of carbohydrate residues, generating a [GlcNAc2Man]2 glycoform, resulted in destabilization of the C_H2 domain while C_H3 domain remained unchanged. To determine the K_D, enthalpy change, and the number of binding sites for the IgG-sFcγRIIb interaction, ITC was carried out at 30 °C. The thermodynamic characteristics for this interaction was enthalpically and entropically favorable, with a binding stoichiometry of 1. The thermodynamic parameters for sFcγRIIb binding to intact IgG molecules were almost the same as those for IgG-Fc, indicating that the Fab regions of IgG do not contribute significantly to binding of FcγRIIb. Interactions between sFcγRIIb and homogeneous glycoforms of IgG-Fc were investigated by ITC. The native Fc and its nongalactosylated glycoform, showed very similar thermodynamic parameters. Removal of terminal GlcNAc residues to generate the (M3N2F)2 glycoform resulted in only a slight decrease in binding affinity without a significant change in enthalpy, indicating a less favorable entropic contribution. However, removing the

terminal mannose residues generated the (MN2F)2 glycoform that showed a marked reduction in binding affinity and enthalpy without a significant change in entropy. Finally, interaction of deglycosylated Fc with sFcγRIIb was not detectable. SPR resulted in different K_D values compared to ITC, however ITC and SPR both showed the same trends in that removal of the terminal GlcNAc and mannose residues of the oligosaccharide chains of IgG-Fc results in a slight and marked decrease in affinity. These results suggest that removal of the oligosaccharides of IgG-Fc causes disorder and a closed disposition of the two C_H2 domains, adversely affecting sFcγRIIb binding. The dramatic reduction of FcγR and C1 binding and activation for aglycosylated IgG-Fc is in opposition with reports of minimal structural change within the protein structure.

Okazaki et al. [37] used ITC to show that fucose depletion from human IgG1 enhanced binding enthalpy and binding association. This was the first published case where a glycoform modification improved glycoprotein affinity for the receptors without carbohydrate-binding capacity, suggesting a potential novel glyco-engineering strategy to improve ligand–receptor binding. ITC demonstrated that IgG1–FcγRIIIa binding was driven by favorable binding enthalpy but opposed by unfavorable binding entropy change. Fucose depletion from IgG1 enhanced the favorable enthalpy, leading to the increase in the binding constant of IgG1 for the receptor by a factor of 20–30. SPR showed that the increase in the binding affinity was due to an enhanced association rate. As a comparison, ITC data using a triple amino acid substitution in IgG1, S298A/E333A/K334A (known to improve IgG1 affinity for FcγRIIIa) demonstrated that the amino acid substitution attenuated the unfavorable entropy resulting in a three- to fourfold increase in the binding constant. These results suggested that the mechanism of affinity improvement by the fucose depletion is distinct from that by the amino acid substitution. The authors also examined the effect of FcγRIIIa polymorphism (Val158/Phe158) on IgG1–FcγRIIIa binding. The Phe to Val substitution increased FcγRIIIa affinity for IgG1 in an enthalpy-driven manner (from ITC) with a reduced dissociation rate (from SPR).

ITC data can complement the structural data achieved with emerging technologies in the characterization of antibodies, including hydrogen-deuterium exchange mass spectroscopy (HDX-MS). HDX-MS uses the intrinsic exchange of hydrogen atoms in a protein to serve as reporters on its local/ global structural environment. Houde et al. first described the use of HDX-MS for routine analysis of monoclonal antibodies [38]. HDX-MS is being incorporated in higher-order structure analysis for biopharmaceutical development, and is used to look at structural differences such as glycosylation during purification.

Carbohydrate/antibody interactions

Cell-surface glycans and polysaccharides have been studied as potential vaccine targets, and anti-carbohydrate antibodies have been developed and characterized with ITC [39,40]. Large glycans are highly antigenic and immunogenic, which is a challenge for designing a selective vaccine, so smaller and less complex antigens which provide a similar immune responses are desirable for vaccine development. Broecker et al. [41] demonstrated that disaccharides multivalently linked on a synthetic OAA scaffold were highly antigenic and induced antibodies to larger *C. difficile* surface Polysaccharide-I (PS-I) glycans in mice. Molecular insights into interactions of purified monoclonal antibodies (mAbs) with mono- and multivalent glycans involved SPR, saturation transfer difference (STD)-NMR and ITC experiments. The binding strengths of the mAbs to PS-I glycans (shown in **Figure 3**) were determined by SPR [41]. K_D values to glycan 1 were around 200 nanomolar, binding to 2 was around 15 micromolar, and binding to 3 was around 15 micromolar. Weak or no binding was observed for glycans 4 and 6. Assuming that 1 had more interactions with the binding pockets of the antibody, the authors thought that more water molecules would be released during complex formation, compared to binding with 3, resulting in a favorable entropic contribution. ITC was used to observe

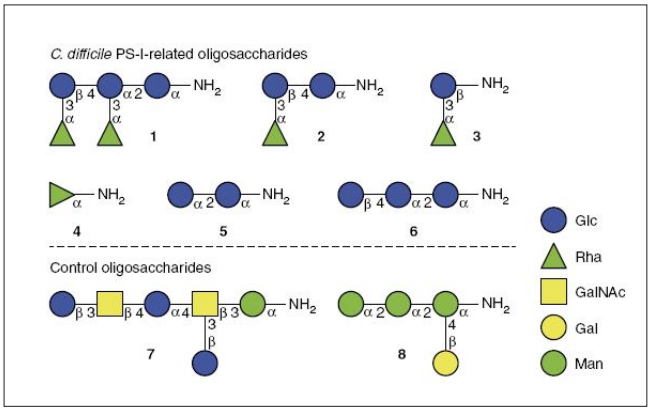

Figure 3. Oligosaccharides used in ITC and SPR binding experiments to study anti-PS- I monoclonal antibodies. Reprinted with permission from Reference [41].

the thermodynamics of monoclonal antibodies binding to glycans 1 and 3 **(Figure 4)**. Both 1–antibody and 3–antibody interactions were mainly enthalpically driven

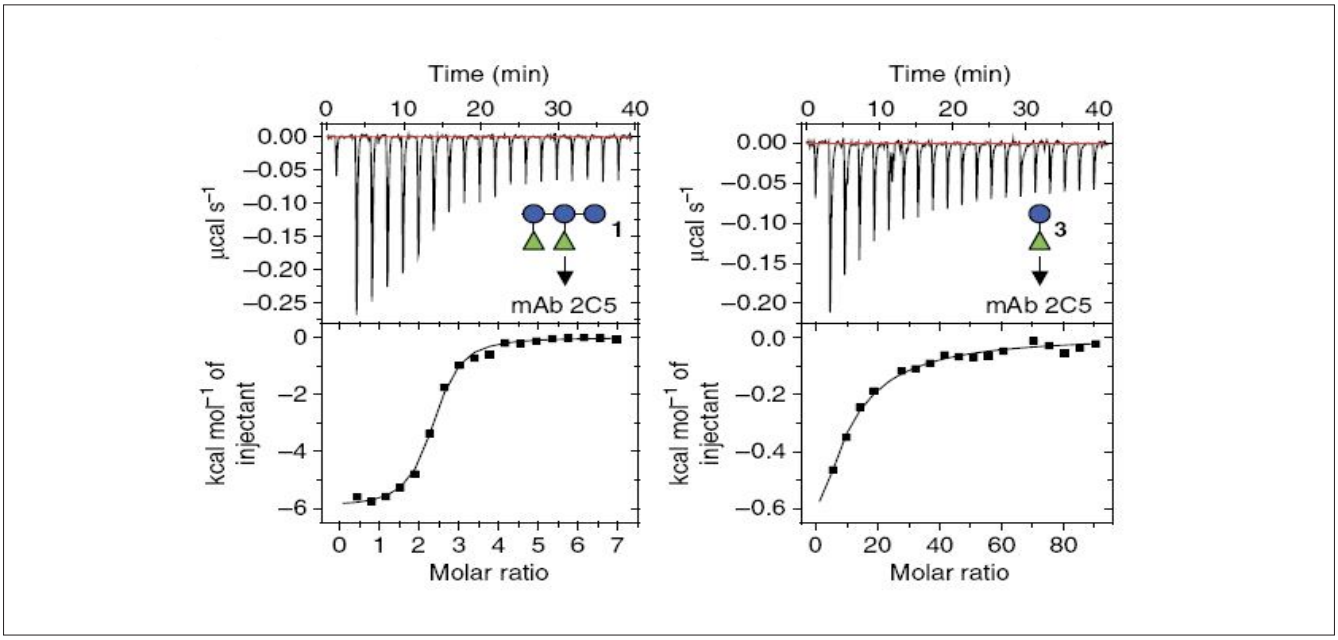

Figure 4. ITC data for binding of mAb 2C5 titrated with glycan 1 (left) or glycan 3 (right). Raw ITC data in top panels, binding isotherms in bottom panels. Reprinted with permission from Reference [41].

(Figure 5). Entropic contributions were favorable for 1, but were lower or slightly unfavorable for 3. Entropically favored binding was likely responsible for the increased affinity to 1, possibly because it provides more hydrophobic interactions through methyl groups of rhamnoses. The favorable entropic terms supported the notion that 1 fills the binding pocket of the mAbs.

Taking all the data together as presented in their article, Broecker et al. had experimental evidence that artificially connecting minimal glycan epitopes were able to mimic larger glycan structures for an immune response. This information could be used to rationally design antigens for vaccines against *C. difficile*.

Therapeutic antibodies

Monoclonal antibodies are among therapeutic protein drugs currently on the market (including several "blockbuster" drugs), and more antibodies are in pre-clinical and clinical trials. Monoclonal antibodies are used as therapies for cancer, inflammatory and autoimmune diseases. ITC is among the biophysical techniques used to characterize bioactivity, as well as the mechanism of action during drug discovery and development.

Doern et al. [42] investigated a panel of antibodies directed against the type 1 insulin-like growth factor receptor (IGF-1R), as possible cancer therapies. Their studies identified four distinct inhibitory classes of antibodies: allosteric blockers of substrate IGF-1 binding; allosteric blockers of substrate IGF-2 binding; allosteric blockers of IGF-1 and IGF-2 binding; competitive blockers of IGF-1 and IGF-2 binding. Using ELISA, SPR, DSC, circular dichroism, immunoprecipitation, and ITC, they mapped the different epitopes, and measured the substrate-receptor binding in the absence and presence of the different antibodies to demonstrate allosteric or competitive blocking. ITC showed the affinity of IGF-1 binding to IGF-1R was temperature-dependent, becom-

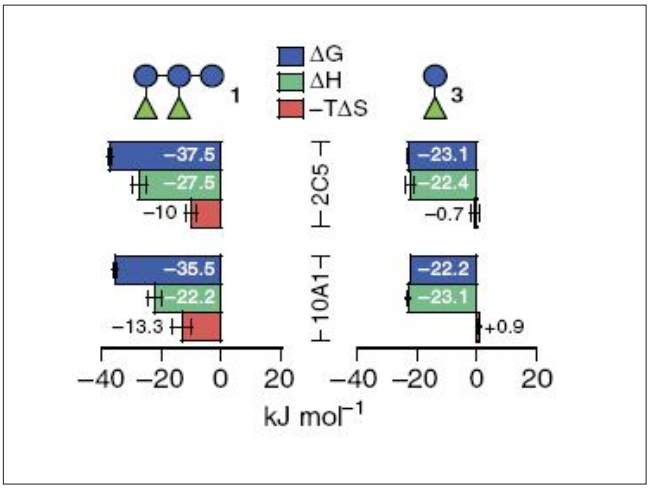

Figure 5. Summary of thermodynamic parameters determined from ITC for glycans 1 (left) and 3 (right) interacting with mAbs 2C5 (top) and 10A1 (bottom). ΔG data are blue bars, ΔH data are green bars, and -TΔS data are red bars. Bars represent mean ± s.e.m. of two independent measurements. Reprinted with permission from Reference [41].

ing weaker as the reaction temperature increased from 5 °C to 37 °C. CD studies on the proteins did not show any structural changes between 5 °C to 50 °C. Entropic penalties associated with antibody-antigen complex and CD data suggests binding of IGF-1 substrate or the inhibitory antibodies was associated with conformational changes in IGF-1R, linked to the ordering of dynamic or unstructured regions of the receptor. These results suggest IGF-1R uses disorder/order within its polypeptide sequence to regulate its activity.

Trastuzumab (brand name Herceptin) is a humanized anti-Epidermal Growth Factor Receptor 2 (HER2) antibody, used as a therapeutic agent for treating breast and gastrointestinal cancers. Most monoclonal antibodies are specific again one antigen. Recent research efforts have been investigating the development of antibodies which are reactive against two or more antigens to be used as therapeutic agents. Bostrom et al. [43] engineered the antigen-binding site of Herceptin and added a second specificity toward Vascular Endothelial Growth Factor (VEGF). This resulted in a high affinity two-in-one antibody bH1. Crystal structures of bH1 in complex with either antigen showed that, bH1 exhibited greater conformational variability, or structural plasticity, compared to Herceptin alone. Using ITC, DSC, SPR and molecular modeling, they analyzed the biophysical and thermodynamic properties of the dual specific variants of Herceptin to gain insight on how a single antibody can bind two unrelated protein antigens. Using SPR, they showed that bH1 and the affinity-improved variant bH1-44, maintained binding affinity, and kinetics when compared to Herceptin. However, ITC showed that the bH1 and variants differed in the Herceptin binding thermodynamics. Her2-bH1 and VEGF-bH1 binding showed large favorable entropy changes, while the Herceptin-HER2 interaction showed a large favorable enthalpy change. The authors showed that the structural plasticity of the bH1 antibodies from the dual specificity did not translate into the expected increase of entropic penalty (relative to Herceptin). It is possible that increasing the structural plasticity of an antigen-binding site, without increasing the entropic cost, can be important for antibodies to develop multi-specificity. This research also contributed to the understanding of the thermodynamics for antigen recognition of the immune response and antibody repertoire.

During the development of new antibody drugs, the protein is engineered to add a desirable feature and/or remove a detrimental feature. For example, Xie et al. [44] evaluated antibodies that could block CD40–CD40L interactions. These antibodies showed a positive clinical effect in treating autoimmune diseases, however some patient developed thromboembolism, the formation of a blood clot, which became dislodged and blocked a blood vessel in the lungs, brain, or other part of the body. This outcome stopped further development of these antibodies. In this study, the authors showed the interaction of the wild-type IgG1 Fc domain of anti-CD40L mAbs activated FcγRIIa (CD32a) receptor on platelets, and this interaction was responsible for platelet aggregation and thromboembolism. Using biophysical techniques such as SPR and ITC, they demonstrated that a mutated IgG1 tail showed minimal FcγR binding and platelet activation while maintaining full binding to CD40L. An Ab fragment, termed a "domain Ab" (dAb), against murine CD40L was identified and fused to a murine IgG1 Fc domain containing a D265A mutation that lacks Fc effector function. The *in vitro* and *in vivo* data showed that immunosuppression and thromboembolism can be "uncoupled" and that a CD40L dAb with an inert Fc tail could be a viable treatment against autoimmune diseases. Biosimilar versions of biopharmaceuticals, are now being developed and approved for commercial use. Biosimilars have to undergo similar characterization as the original products, possibly including ITC results [45].

HIV neutralizing antibodies

Development of therapies against HIV have involved neutralizing antibodies, including those against the HIV-1 envelope glycoprotein gp120, and several studies incorporated ITC in the characterization of the antibody-target interaction [46-52]. The high-mannose patch on the HIV-1 envelope (Env) glycoprotein is a key component for binding of broadly neutralizing PGT121 family of antibodies, however ways to generate such antibodies by vaccination are not currently defined. Garces et al. [53] generated clones and structures of antibody intermediates at different stages of maturation to determine the molecular events that occurred during evolution of this antibody family. Site directed mutagenesis, combined with ITC, revealed that affinity maturation involved the N137 glycan. During the maturation process, binding affinity was differentially gained (become tighter affinity) in each of the two major branches of the PGT121 family. The authors propose that some variation evolved in the PGT121 family branches that led to differences in glycan specificities in their respective epitopes. Thermodynamic and structural insights can help advance the design of HIV vaccine candidates.

Beyond monoclonal antibodies

There is a growing interest in generating recombinant

affinity reagents as an alternative to traditional antibody production in animals or cell lines. These would be used in basic research as well as potential therapeutic agents. These affinity reagents are genetically fused to other proteins or epitope tags, which can then be used for conjugation to other modules for research or diagnostic work. Another important feature is that their affinity and specificity can be controlled. Display technologies are used to screen libraries by affinity selection, yielding clones that are specific and high-affinity for their cognate targets. One such scaffold classification is "nanobodies," which are single-domain fragments of camelid antibodies [54]. Nanobodies are emerging as versatile tools in biotechnology. Several recent studies incorporated ITC to characterize nanobodies function and structure [55-57].

De Genst et al. [58] developed a nanobody NbSyn2, selected by phage display techniques to bind to α-synuclein. Aggregation of α-synuclein is associated with neurological disorders, including Parkinson's disease, and the goal of this study was to develop a nanobody probe to that can be used to understand the molecular mechanism of α-synuclein aggregation. The authors used ITC and observed that NbSyn2 specifically bound to monomeric α-synuclein with nanomolar affinity, and NMR spectroscopy showed NbSyn2 interacted with the four C-terminal residues of the protein. This latter finding is confirmed by the determination of a crystal structure of NbSyn2 bound to a peptide encompassing the nine C-terminal residues of α-synuclein. The NbSyn2: α-synuclein interaction is mediated by side-chain interactions, while water molecules cross-link the main chain atoms of α-synuclein to atoms of NbSyn2. The aggregation behavior of α-synuclein at physiological pH, including the morphology of the resulting fibrillar structures, was surprisingly unaffected by the presence of NbSyn2. The authors used ITC to show that NbSyn2 tightly bound to the aggregated as well as to the soluble forms of α-synuclein. These results suggest that binding of NbSyn2 could be a useful probe for the identification of α-synuclein aggregation in vitro and possibly in vivo.

Conclusions

The demand for the development and production of antibodies for use in basic research, diagnostics, vaccine development, and biotherapeutic drugs will continue to grow. Antibody design and engineering require a detailed understanding of the binding, specificity, thermodynamics, and structure of the antibody-antigen interactions, which can be used to guide the production of antibodies with specific functions and features. New products such as bi-specific antibodies, nanobodies, and antibody-drug conjugates are being introduced. ITC is a key biophysical characterization tool and will continue to be a core assay for antibody analysis during research, discovery, and development.

While monoclonal antibodies used for clinical studies and as drug products are thoroughly validated for activity, specificity and selectivity by biophysical and biochemical assays, that is not the case for commercially available antibodies sold as protein-binding assay reagents. There have been recent discussions in the scientific community about the lack of standardization for antibodies used for research [59] where it is believed that poorly characterized antibodies can account for poor reproducibly of research studies. Since ITC is a precise measurement of the specific interaction, ITC can be incorporated as routine standardization test for commercial antibodies.

ITC hardware and software will also continue to improve, resulting in instruments generating faster results, using less sample, providing binding and thermodynamic data with better reliability. ITC will continue to play a vital role in antibody research and biopharmaceutical discovery and development.

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Citation:
Frasca V. Biophysical characterization of antibodies with isothermal titration calorimetry. J Appl Bioanal 2(3), 90-102 (2016).

Funding/Manuscript writing assistance:
The author has financial support or funding to report and she also declares that no writing assistance was utilized in the production of this article.

Competing interest:
The corresponding author is employed (full time) by Malvern Instruments.